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CHARACTERIZATION AND STABILIZATION OF *CLOSTRIDIUM BOTULINUM* NEUROTOXIN FOR MEDICAL USE

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CHARACTERIZATION AND STABILIZATON OF *CLOSTRIDIUM BOTULINUM* NEUROTOXIN FOR MEDICAL USE

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MICHAEL CHARLES GOODNOUGH

A thesis submitted in partial fulfillment of the
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...and all this science I don't understand. It's just my job five days a week.

-Elton John, *Rocket Man*

CHARACTERIZATION AND STABILIZATION OF *CLOSTRIDIUM BOTULINUM* NEUROTOXIN USED MEDICALLY

Michael Charles Goodnough
Under the supervision of Associate Professor Eric A. Johnson
at the University of Wisconsin-Madison

Clostridium botulinum produces one of the most potent neurotoxins known. This neurotoxin causes a flaccid paralysis by preventing the release of the neurotransmitter acetylcholine across the synaptic junction of the motor endplate. The neurotoxin is the causative agent of foodborne botulism, wound botulism, and infant botulism. In the past 10-15 years, the neurotoxin has been developed for use as a therapeutic agent in the treatment of focal dystonias and spastic muscle disorders by direct injection of nanogram quantities into overactive muscles. In this research, I have addressed issues related to the quality of botulinum toxins for medical use.

Differentiation of individual neurotoxin serotypes was accomplished by development of a sensitive colony immunoblotting assay that was capable of detecting toxin producing colonies as well as distinguish between high titer colonies and those producing less neurotoxin. Neurotoxin produced by colonies of Clostridium botulinum types A, B, E and toxigenic Clostridium butyricum bound to nitrocellulose was detected by an enzyme-linked immunoassay procedure. The procedure used serotype specific rabbit IgG as the primary antibody and goat antirabbit IgG antiserum labeled with alkaline phosphatase as the marker to visualize immobilized neurotoxin from individual colonies. The method differentiated the colonies based on serotype of neurtoxin produced and by the amount of neurotoxin produced by individual colonies on the same agar plate. Specificity of primary antibodies used was improved by adsorption of cross-reacting heterologous antibodies.

Neurotoxin associated with non-toxic proteins in high molecular weight complexes was characterized at each step in the purification process showing that the purification

process currently approved by the United States Food and Drug Administration is variable with regard to yield and specific toxicity. The toxin for medical use in the United States is produced by the Hall A strain as part of a complex of at least six other proteins and is purified by a series of precipitations and crystallizations. In this study, the toxin complex was examined at each step in the purification scheme for toxicity recovered, % solids, optical density at 260 and 278 nm, specific toxicity, and for the presence of ribonucleic acids. The results show that the procedure is variable and dependent to a large degree on the individual components making up the growth medium indicating nutritional regulation of toxin formation. Ribonucleic acid associated with the purified toxin complex was found at a level of 0.3% and did not appear to be specific.

Botulinal neurotoxin must be lyophilized or freeze-dried to allow for shipping and handling of the relatively delicate protein. Recovery of type A and B toxin activity following lyophilization was dependent on a number of variables. Conditions were found that gave >90% recovery of the toxicity following lyophilization of solutions containing 20-2,000 mouse 50% lethal doses. Full recovery of type A and B toxin complex toxicity as well as the purified ca. 150 kDa toxin molecule was obtained on drying 0.1 ml when the pH was maintained below 7.0 and serum albumins or other protein excipients were used as stabilizers in the absence of sodium chloride. Shelf stability was improved by addition of trehalose to the serum albumin system but not by addition of sucrose or maltotriose. This drying formulation allowed storage of lyophilized type A toxin complex and purified type A neurotoxin at temperatures up to 37°C for months with minimal losses in activity.

Inactivation events which occurred during drying of type A and B neurotoxin were investigated. The various drying processes and formulations cause varying degrees of inactivation of the toxin and formation of toxoid. This toxoid adds to the antigenic burden of the material and further increases the chances of patients developing neutralizing antibodies. The processes which result in the formation of this toxoid can involve

aggregation, deamidation, peptide bond hydrolysis, and oxidative degradation. In this study we demonstrated that in our model system using purified type A and B Clostridium botulinum neurotoxins, aggregation, deamidation, and peptide bond hydrolysis occur during the lyophilization procedure causing decreases in the specific toxicity. Among the events causing loss in specific activity of the neurotoxin, peptide bond hydrolysis at the aspartate12-proline13 residue in the light chain was shown in purified type A neurotoxin. Deamidation was shown to occur in both type A and B purified neurotoxins using radiolabel incorporation with protein isoaspartyl methyl transferase. Type B showed more deamidation than type A neurotoxin. Aggregation also caused losses of both neurotoxin serotypes during lyophilization.

Sub lethal doses of various preparations of Clostridium botulinum type A toxin including Botox®, and Dysport®, were tested in a rabbit model for immunogenicity. Known quantities of various type A toxin preparations were injected over a period of time and the animals serum assayed for antibodies capable of neutralizing a small challenge of purified type A toxin in mice. Animals injected with Botox® developed neutralizing antibodies to the toxin in 60 and 63 days. Animals injected with type A toxin complex produced in our laboratory produced antibodies that neutralized the challenge in 118 days while animals treated with purified type A neurotoxin and Dysport® did not produce neutralizing antibodies over the course of the 2-3 month injection schedule.

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Chapter I

Introduction

Members of the genus Clostridium produce some of the most potent neurotoxins known. Within this genus, the species Clostridium tetani and Clostridium botulinum are probably the most widely known. Neuropoisonings due to these organisms have been recognized since antiquity. Hippocrates described a case of tetanus as follows: "The master of a large ship crushed the index finger of his right hand with the anchor. Seven days later a somewhat foul discharge appeared; then trouble with his tongue-he complained that he could not speak properly...his jaws became depressed together, his teeth were locked, then symptoms appeared in his neck; on the third day opisthontus appeared with sweating. Six days after the diagnosis was made he died." Although caused by a similar neurotoxin, botulism is more subtle in its symptoms. In contrast to the spastic paralysis of tetanus, botulism causes a flaccid paralysis which may have gone undetected for many years as a disease. Niemann (1991) quotes an author of a textbook on poisons named Schanaq who described the production of a highly potent toxin: "Collect blood from the left vein of the neck of a black bull, fill it into an unrinsed sheep gut, seal it tightly and dry the content in the shade of a mulberry tree. The powdered residue intermingled with food will lead to death within three days." This author may have described the first commercial drying operation of botulinum toxin as well!

Botulinum toxin has been used therapeutically since the early 1980's in the treatment of spastic muscle disorders thanks in large part to the efforts of Drs. Edward J. Schantz and Alan B. Scott. My research into the nature and stabilization of botulinal neurotoxins is organized into eight chapters. The first is a brief, historical introduction. The second chapter is a review of the current literature. It is meant to give the reader a general background on the subject of Clostridium botulinum and it's neurotoxin. It is by no means exhaustive. Further, more specific background information is presented in the introduction of the five succeeding chapters describing my research. These five chapters (III-VII) describe original research and are arranged in the format of research articles.

Material from chapters III and V was previously published in Applied and Environmental Microbiology while portions of chapter V have been accepted for publication by the American Chemical Society in a symposium series book. There is information in both chapters that has not been previously published.

Chapter III deals with detection and differentiation of botulinum neurotoxin in and around colonies grown on agar medium. Chapter IV describes the characterization of the type A botulinum toxin purification as done by Schantz for production of 79-11 and other lots of pharmaceutical grade toxin. Chapter V describes research on the stabilization of some of the various botulinum neurotoxins during lyophilization. Chapter VI deals with some of the possible mechanisms of inactivation that occur when toxin is lyophilized. Chapter VII describes an animal model for testing the immunogenic potential of different botulinum toxin preparations including two commercially available products. Finally, Chapter VIII contains conclusions and future prospects along with some commentary that did not seem to fit any other place in this thesis.

Reference:

Niemann, H. 1991. Molecular biology of clostridial neurotoxins. *In* Sourcebook of bacterial protein toxins, J. Alouf and J. Freer (eds.). Academic Press Ltd., London.

CHAPTER II

Background and Significance

Classification.

Bergey's Manual (Cato et al., 1986) classifies Clostridium botulinum as a gram-positive, obligately anaerobic, endospore forming rod-shaped organism with general dimensions of ca. 1.5-2.0 x 3.0-6.0 μm. The organisms that are classified as C. botulinum have in common the characteristic ability to produce a very potent neurotoxin. This neurotoxin is proteinaceous in nature and acts on the pre-synaptic junction of motor-neurons and the muscle fibers innervated by the neuron. Botulinum neurotoxin is quantitated in terms of mouse intraperitoneal 50% lethal doses (LD_{50}). (Schantz and Kautter, 1978).

There are at present seven known serotypes of botulinum neurotoxin, A, B, C_1, D, E, F, and G. Two additional toxins are produced by some type C strains, C_2 and C_3 toxin, which are classified as cytotoxins due to their modes of action. These latter two clostridial toxins are ADP-ribosylating toxins as opposed to neurotoxins (Aktories et al., 1986; Moriishi et al., 1991). Generally, a given strain produces a single neurotoxin type but there are exceptions. Some strains have been known to produce two serotypes of toxin. One, type A_f, produced 93% type A toxin and 7% type F toxin while another designated B_f produced 90% type B toxin and 10% type F (Hatheway, 1989). Types C and D strains are known for interconversion of toxin serotypes by phages (Smith and Sugiyama, 1988).

Other clostridia have been isolated that produce botulinum-like neurotoxins. An organism phenotypically resembling C. baratii was isolated in 1985 from a patient in New Mexico with infant botulism (Hall et al., 1985). This organism produced type F neurotoxin and was determined by DNA homology to be a toxigenic strain of C. baratii (Suen et al., 1988). Two separate cases of infant botulism in Italy were determined to have

been caused by an organism that phenotypically closely resembled C. butyricum but was capable of producing botulinum neurotoxin (McCrosky et al., 1986; Aureli et al., 1986). Again, DNA homology showed the organism to be C. butyricum (Suen et al., 1988).

C. botulinum strains are further classified phenotypically by proteolytic activity. Group I organisms are those that digest coagulated egg white or meat particles and are considered to be proteolytic as well as saccharolytic; Group II organisms are those that are considered non-proteolytic and saccharolytic; Group III consists of those organisms that may or may not be proteolytic and produce one or more of the toxin serotypes mainly considered to affect birds and animals; while Group IV organisms are designated non-proteolytic and non-saccharolytic type G strains. A summary of the various serotypes and their culture group is shown in the following (adapted from Sugiyama and Sofos, 1988, and Rhodehamel et al., 1992):

Group I (proteolytic saccharolytic strains) All type A strains, proteolytic type B and F strains.	Spores Oval and subterminal
Group II (non-proteolytic, saccharolytic strains) All type E strains, non-proteolytic type B and F strains.	Oval and eccentric to subterminal
Group III (proteolyic or non-proteolytic strains producing one or more of the following toxin serotypes) Type C_1, C_2, C_3, and D.	Oval and subterminal

Group IV	
(non-proteolytic, non-saccharolytic strains)	Oval and subterminal
All type G strains.	

Group I and II strains are the predominant causes of human botulism while Group III strains are primarily known for causing avian botulism (Smith, 1982) as well as botulism of cattle, sheep (Smith, 1977) and some captive animals such as lions (Greenwood, 1985).

Growth requirements.

Most research attention has been focused on the growth of C. botulinum in foods for obvious reasons. The predominant factors involved are temperature, pH, water activity, redox potential, presence or absence of inhibitors and competing microflora. Most processed foods present a series of these "hurdles" acting together to prevent outgrowth of the organism and toxin formation.

Temperature requirements vary between the different groups. Group I organisms typically have a growth optimum of 37°C and a minimum of ca. 10°C with a maximum of 45-50°C. Group II organisms which are considered to be psychrotrophic and have growth optima of ca. 30°C; they are capable of growth and toxin formation at temperatures as low as 3.3°C. Maximum growth temperatures for group II organisms are in the range of 40-45°C (Hauschild, 1989).

Lower pH limits for growth of group I strains have been found to vary in the range of 4.6-5.0. For group II strains the limit is ca. 5.0. These limits are dependent on all other factors being close to optimum. The upper limit of growth for C. botulinum has been reported to be in the range of pH 8-9 (Hobbs, 1976).

As with all organisms, growth of C. botulinum depends on a minimum quantity of available water (Aw). Water activity in foods is usually controlled by the use of salt

(sodium chloride) or sugars. With other conditions such as pH, temperature, etc. being close to optimum, the maximum salt concentrations for growth of group I and II organisms is in the range of 10% (Aw = 0.94) for group I and 5% (Aw = 0.97) for group II (Genigeorgis, 1986).

Redox potential levels that will allow the initiation of growth of C. botulinum are quite high for an obligate anaerobe, being on the order of +200 mv if there are no other environmental stresses. One of the primary methods of inhibition of C. botulinum in cured meats is inclusion of nitrite and nitrate. Nitrate is reduced to nitrite which is the active compound. Nitrite is believed to react with iron-sulfur proteins such as ferredoxin to form iron-nitric oxide complexes. These claims are supported by the work of Tompkin et al. (1978). In their work, the addition of iron salts or iron ions counteracted the inhibitory effect of nitrite while addition of iron-chelating compounds enhanced the nitrite effect. Nitrite has the additional attributes of giving cured meats their pink coloration as well as acting as an antioxidant (Rhodehamel et al., 1992).

The presence of competitive microflora can allow the food processor to use lower levels of other preservative compounds such as nitrites in semipreserved meats. Lactic acid bacteria are generally added to the product along with a fermentable carbohydrate such as glucose or sucrose. Temperature abuse of the product promotes rapid growth of the competitive microflora and fermentation of the carbohydrate with a concomitant drop in the pH of the system. Some lactic acid bacteria also produce bacteriocins which have been shown to be inhibitory to C. botulinum under some conditions (Tanaka et al., 1985).

Thermal resistance of C. botulinum endospores.

Endospores of C. botulinum are of critical importance to food industries due to their very high thermal resistance. Spores of group I C. botulinum are much more heat-stable than those of group II . Consequently, most work involving thermal processing and D and

Z values has been done with group I organisms. A D-value is equal to the heat-treatment that results in a 90% reduction in the bacterial population of interest. A Z-value is the change in the temperature that results in a 10-fold change in the D-value. The minimum thermal process that is applied to low-acid (pH >4.6), non-refrigerated, commercial foods is called a 12D process. This process theoretically reduces a load of 10^{12} viable endospores to 10^{0}. Since the hypothetical 10^{12} spore load is much higher than any seen in the canning industry, such a process ensures that there are no viable C. botulinum spores remaining in the food (Rhodehamel et al., 1992). D121°C values for group I spores have been reported to be 0.03-0.23 min (ICMSF, 1980).

The ability of group II organisms to grow at refrigeration temperatures is important due to the fact that many refrigerated foods do not receive a 12D thermal process but instead are pasteurized and then refrigerated. Because of this fact, endospores of group II organisms are usually tested for thermostability at 82°C. Non-proteolytic type B spores have reported D82°C values of 1.5-32 min while other group II organisms such as type E strains and non-proteolytic type F strains are in the D82°C 0.2-1.0 min range (Hauschild, 1989).

Distribution of C. botulinum.

The endospores of C. botulinum are found worldwide in soil samples and in freshwater and marine sediments (Rhodehamel et al., 1992). The distribution of in the United States is such that type A C. botulinum occurs more frequently in the western U.S. while type B occurs more commonly in the eastern U.S. Type E spores are often associated with marine and freshwater sediments.

II. The disease.

Foodborne botulism.

The disease botulism is classically associated with the consumption of preformed toxin in contaminated foods. Originally, blood-sausages were known to be a cause of the disease. The Latin word for sausage is *botulus*. The disease was first reported by van Ermengem in 1897 after an investigation of an outbreak in Belgium in 1895 (van Ermengem, 1979 translation). The causative organism was recovered from inadequately cured, unsalted ham and had caused 13 cases of botulism, 3 of which were fatal. The organism was named Bacillus botulinus by van Ermengem. van Ermengem showed that culture filtrates of this organism caused botulism in various animals and that the agent was heat labile and stabilized by acid. Since the time of van Ermengem, botulism has been associated with many other types of foods as well as with other disease etiologies. In the United States, the primary source of foodborne botulism is the consumption of improperly canned vegetables. Recently, the largest outbreaks of botulism in the United States have involved restaurants. In one the implicated food was improperly canned jalapeno peppers (Terranova et al., 1978); in another potato salad was implicated (Seals et al., 1981); and in yet another the toxin was formed in sautéed onions (MacDonald et al., 1985). Most of the foodborne outbreaks of botulism in the continental United States are caused by type A or B producing strains of C. botulinum while 32 of 44 outbreaks in Alaska since 1944 have been caused by type E C. botulinum (Wainwright et al., 1986). Type E C. botulinum is usually associated with fish or other marine-related foods such as dried and smoked fish, fermented whale blubber, and seal meat stored under seal oil (Wainwright et al., 1986).

Wound botulism.

A much rarer type of botulism is caused by C. botulinum contamination of a wound with subsequent growth of the organism and production of the toxin *in vivo* rather than ingestion of the preformed toxin. This type of botulism is termed wound botulism and is analogous to the disease caused by a close relative of C. botulinum, namely C. tetani.

Infant botulism.

Infant botulism is currently the leading cause of botulism in the United States. This disease is caused by the ingestion of endospores and growth of C. botulinum in the infants gut with subsequent absorption of the toxin through the intestinal barrier. In the United States between 1975 and 1991 there were a total of 1013 cases reported to the CDC of which 480 were caused by type A C. botulinum and 522 were caused by type B C. botulinum (Rhodehamel et al., 1992). In a study of 336 patients with infant botulism, correlation has been demonstrated between toxin from stool samples and the presence of culturable C. botulinum (Hatheway and McCroskey, 1987).

III. The etiological agent.

C. botulinum toxin complex.

Toxins of the different C. botulinum serotypes are usually produced in culture as aggregates of neurotoxin and other non-toxic proteins associated into a polypeptide complex (Schantz, 1964; Sugii and Sakaguchi, 1975; Kozaki et al., 1974; Miyazaki, et al., 1977; Kitamura et al., 1969; Ohishi and Sakaguchi, 1974; Yang and Sugiyama, 1975; Nukina et al., 1987). These toxin complexes vary in size from ca. 900,000 daltons for type A LL toxin complex (Schantz, 1967) to ca. 300,000 daltons for the type B M complex (Kozaki et al., 1974) and type E complex (Kitamura et al., 1969), to 235,000 daltons for type F M complex (Ohishi and Sakaguchi, 1974). Descriptions of the sizes of toxin complexes has been confusing since various methods have been used in these determinations. Molecular weight determinations of the various sized complexes in the 1960's through the 1970's was done with ultracentrifugation and sizes were expressed in terms of Svedberg units. The results of some of these studies are shown in Table 1 (adapted from Sugiyama, 1980). Toxin complexes are described as M for medium, L for large and LL for very large. According to Sugii and Sakaguchi (1977), during culture the proportion of one toxin complex vs. another is dependent on the growth medium and conditions. A type B culture grown in the presence of 1 mM Fe^{+2} produces an equal proportion of L and M complexes while the same culture grown in the presence of 10 mM Fe^{+2} produces predominantly M complex.

Table I. Molecular sizes of various C. botulinum toxin complexes.

Toxin type	Sedimentation coefficient	ca. M_r (kDa)
LL A	19S	900
L A, B, D	16S	450-500
M A, B, C_1, D, E, F	10-12S	235-350

Some of the non-toxic proteins associated with the various toxin complexes have hemagglutinating abilities (Sugiyama, 1980; Somers and DasGupta, 1991). In particular, non-neurotoxic fractions of the L complexes of type A, B, and D have been shown to have hemagglutininating activity. Hemagglutinin fractions isolated from the different serotypes show some serological cross-reactivity. Non-toxic fractions from type A and B serotypes cross-react (Goodnough and Johnson, 1993) as do non-toxic fractions from types E and F. The non-toxic fractions of types C_1 and D are antigenically identical as determined by Ouchterlony diffusion (Sakaguchi et al., 1974).

The non-toxic complexing proteins have been demonstrated to be essential for stabilization of the toxin during passage through the digestive tract (Ohishi and Sakaguchi, 1974; Sakaguchi et al., 1981). Pure neurotoxin has a peroral LD_{50} about 100-10,000 times lower than that of toxin complex on a weight basis (Ohishi, 1984, Sakaguchi, 1983). Presumably, the complexing proteins protect the very labile toxin molecule from proteolytic hydrolysis and other means of inactivation by enzymes present in the gut and circulatory systems since the toxin and the complexing proteins are very stable in low pH environments. The relationship between the size of the toxin complex and the peroral

LD_{50} is such that the larger the toxin complex the lower the LD_{50} indicating that there is a protective effect occurring in the gut. The nontoxic proteins may also promote absorption of the neurotoxin across the intestinal barrier. Following absorption, the neurotoxin component occurs free in the lymph and blood of animals fed toxin complexes (Heckley, 1960; Sugii et al., 1977).

Analysis by SDS-PAGE has shown that type A toxin complex consists of seven different nontoxic proteins ranging in size from ca. 17,000 daltons to 118,000 daltons in association with a neurotoxic protein of ca.147,000 daltons (Gimenez and DasGupta, 1993; DasGupta, 1980; Goodnough and Johnson, 1993, in press). Isolated type A toxin complex has a specific toxicity of 2-4 x 10^7 intraperitoneal LD_{50}/mg in 18-22g white mice. Specific toxicities of other C. botulinum toxin complexes are type B M complex- 4-9 x 10^7 LD_{50}/mg, type C_1 M complex- 3 x 10^7 LD_{50}/mg, type D M complex- 8 x 10^7 LD_{50}/mg, type E M complex- 1 x 10^7 LD_{50}/mg, type F M complex- 2-3 x 10^7 LD_{50}/mg (Sugiyama, 1980), and 8-9 x 10^6/mg for type G (unpublished data).

C. botulinum neurotoxin.

The active neurotoxin of C. botulinum is a dichain molecule of ca. 150 kDa in molecular weight. The molecule is composed of two fragments or chains that are termed the heavy chain (Hc, ca. 100 kDa) and the light chain (Lc, ca. 50 kDa) that are connected by one disulfide linkage. The neurotoxin is synthesized by the organism as a single polypeptide and undergoes posttranslational processing termed nicking to generate the two separate chains by at least one protease (Figure 1) (Yokosawa et al., 1986; Krysinski and Sugiyama, 1981). The nicking event occurs in the culture fluid for proteolytic C. botulinum and through the activity of an exogenous enzyme such as trypsin in non-proteolytic strains (Yokosawa et al., 1986, DasGupta, 1990; Kozaki, 1985). Various sizes and properties of botulinal neurotoxins are shown in Table 2.

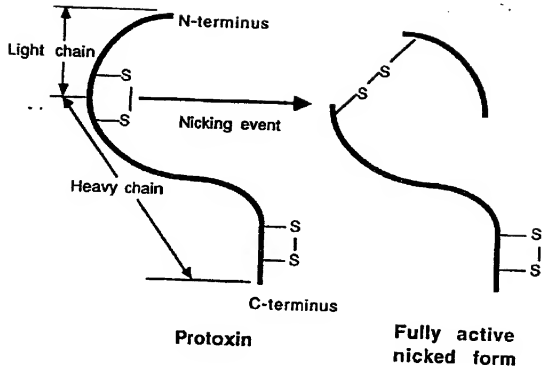

Figure 1. Activation of C. botulinum neurotoxin forming separate heavy and light chains.

Table 2. Properties of various C. botulinum neurotoxins (adapted from DasGupta, 1983; Smith and Sugiyama, 1988).

Serotype	M_r (kDa) Heavy chain	M_r (kDa) Light chain	Extinction coefficient $E^{0.1\%}_{278}$	pI	LD_{50}/mg protein
A	93	52	1.63	6.1	1.05×10^8
B	101	53	1.85	5.25	1.14×10^8
C_1	98	53	1.42		4.3×10^7
D	85	56			
E	100	55	1.63		6×10^7
F	105	56		5.7	2.5×10^7
G					$2\text{-}4 \times 10^7$

Non-chromatographic methods.

Crude purification of botulinum toxin was first demonstrated by Snipe and Sommer (1928) when they showed that 90% of the toxic material in a pure culture of C. botulinum could be recovered by addition of acid to pH 3.5. The first reported purifications of type A botulinal toxin that recovered a quantity of material of sufficient purity for crystallization was that of Lammana et al. (1946). This method utilized a step that included shaking the extracts of the acid precipitated material with chloroform. The method was further amended by Duff et al. (1957) who included precipitation of extracted toxin with ethanol as a means of removing nucleic acids. This method as modified by Schantz (1964) is the method that was approved by the U.S. Food and Drug Administration for production of type A toxin complex for use in humans. This method was selected over others because it is relatively simple and straight forward; it avoids exposure of the toxin to substances such as enzymes or resins from columns and it yields sufficient material for use on a commercial basis (Schantz and Johnson, 1992). This method is described in more detail in chapter III of this thesis, "Characterization of type A Clostridium botulinum toxin complex during purification."

Chromatographic methods.

Column chromatography was first used in the purification of type A and B botulinum toxin by DasGupta and Boroff (1968). This method yielded purified neurotoxin from crystalline type A toxin complex using ion-exchange chromatography and showed for the first time that the specific activity of purified type A neurotoxin was ca. 10^8 LD_{50}/mg. Anion-exchange on DEAE-Sephadex A50 (Pharmacia, Piscataway, NJ) and gel-filtration chromatography (Sephadex G-200, Pharmacia) were used in the early 1970s to purify

toxin from culture fluids without first crystallizing the toxin (DasGupta et al., 1970; Sugii and Sakaguchi, 1975; Ohishi and Sakaguchi, 1977). Others used gel-filtration to obtain partially purified type B toxin complex from culture fluids (DasGupta and Sugiyama, 1976). Type A neurotoxin is now generally purified according to the method of Tse et al. (1982).

With the introduction of different types of ion-exchange resins, cation-exchange was used to further purify neurotoxins that had been partially separated from the non-toxic proteins of the complex (DasGupta et al., 1970; Sugiyama et al., 1974; DasGupta and Sathyamoorthy, 1984; DasGupta and Sugiyama, 1976). SP-Sephadex C50 (Pharmacia) is the most commonly used matrix for this type of chromatography. This type of ion-exchange matrix is not suitable for initial separation of the neurotoxin from the non-toxic complex proteins because of the fact that pH values under 7 are utilized at which the complex is stable. Neurotoxin is separated from most complex proteins at slightly alkaline pH values (DasGupta and Boroff, 1968). An alternative method of separating neurotoxin from complex proteins is through the use of affinity chromatography (Moberg and Sugiyama, 1978). Toxin complex adsorbs to p-aminophenyl-ß-D-thiogalactopyranoside-Sepharose at acidic pH. The neurotoxin is then eluted from the column at alkaline pH with sodium chloride. Type E neurotoxin has been successfully purified from the non-toxic proteins of the complex using high-pressure liquid chromatography (Schmidt and Siegel, 1986). Fast protein liquid chromatography has been used to purify types A, B, and E neurotoxins on anion and cation exchange columns (Woody and DasGupta, 1988).

Biochemical characterization of botulinal neurotoxins.

The neurotoxin of type A botulinal toxin is a protein of ca. 147 kDa comprised of 1295 amino acids (Binz et al., 1990). Two peptide bonds may be cleaved during processing releasing a tetrapeptide (DasGupta and Dekleva, 1990). The nicking occurs approximately

one-third of the distance from the N-terminus of the protoxin molecule removing four amino acids and generates the neurotoxic Hc and Lc connected by at least one disulfide bridge. In type A neurotoxin, the Hc and Lc have molecular weights of ca. 93,000 and 52,000 daltons, respectively (Gimenez and DasGupta, 1993) which corresponds to the predicted molecular weights from the nucleotide sequence of the neurotoxin gene (Binz et al., 1990; Niemann, 1991). The two chains are connected by a disulfide bond between cysteine residues 430 and 454 (Gimenez and DasGupta, 1993; Binz et al., 1990). Reduction of this disulfide bond by sulfhydryl reducing agents such as dithiothreitol or mercaptoethanol generates the separate Hc and Lc. These individual fragments are non-toxic alone but recover toxicity when recombined under appropriate conditions (Maisey et al., 1988). There is a substantial degree of homology between the Hc and Lc chains of the different botulinum serotypes as well as to tetanus toxin (Niemann, 1991; Whelan et al., 1992). The overall percentage identity between the amino acid sequences of the genes for the Lc from tetanus toxin and Lc from botulinum toxin types A, B, C_1, D, and E range from 32 to 51%. There are regions in the various neurotoxins that are strictly conserved many of which probably contribute to biological function. In the H chain, 110 amino acids of ca. 845 total are strictly conserved, and in the L chain 68 amino acids of the ca. 442 total are conserved (Niemann, 1991; Whelan et al., 1992). The availability of the complete amino acid sequences has also revealed the presence of a highly conserved region of hydrophobicity in the H chain, possibly involved in membrane fusion and transport, and also a zinc binding motif in the L chain of serotypes A, B, D, E, and F putting these neurotoxins into the category of zinc metalloendoproteases (Schiavo et al., 1992a; Schiavo et al., 1992b).

Mechanism of action of C. botulinum neurotoxin.

The target of botulinal neurotoxin is the presynaptic junction of motor neurons. The proposed three step mechanism involves binding of the neurotoxin to the target cell surface, uptake or translocation across the cell membrane and internalization via endocytosis, followed by the inhibition of release of the cholinergic neurotransmitter (Simpson, 1981, Niemann, 1991). The receptor itself has not been identified but may involve polysialylated gangliosides or a specific protein (Niemann, 1991). Niemann also suggested that there are at least two different types of receptors with differing affinities. The low-affinity gangliosides are present in abundance (Critchley, et al., 1988) while the presence of a high-affinity protein receptor that is comparatively rare has been suggested by others (Yokosawa et al., 1989; Evans et al., 1985). This work was done with rat-brain and spinal cord membranes under physiological conditions. The existence of high-affinity protein receptors has been questioned, however, since pathological effects of botulinal neurotoxin on brain tissue have never been observed *in vivo* after systemic administration (Niemann, 1991). The binding observed *in vitro* may be due to exposure of protein receptors to the heavy chain of the neurotoxin during preparation of the membranes (Niemann, 1991).

The Hc in all cases is believed to be the binding and internalization trigger for the toxin molecule while the Lc catalyzes the inhibition of synaptic vesicle release when internalized by the target cells (Simpson, 1989). Lc is not taken up by neuronal cells alone and Hc has no neurotoxic effects without being linked to the Lc (Maisey et al., 1988). Chimeric toxins have been created using the Hc of botulinum toxin and the Lc of tetanus toxin that show binding to the neuronal target of botulinal toxin, and paralysis by a mechanism similar to tetanus toxin (Weller et al., 1991). Binding to receptors is believed to be mediated by the C-terminal half of the Hc (Niemann, 1991; Kozaki et al., 1989; Motecucco, 1986), while the N-terminal half of the Hc facilitates passage of the Lc through

cellular membranes (Niemann, 1991; Blaustein, 1987). Montecucco (1986) presented a model for binding of botulinal neurotoxins to neuronal membranes. He proposed that the initial recognition site of the toxin on the neuronal membrane is a ganglioside of the G_{1b} series. The binding of botulinal toxins and tetanus toxin to gangliosides exclusively is not substantiated by certain pieces of evidence that show binding is trypsin sensitive and that radiolabelled tetanus heavy chain can not be displaced by unlabeled heavy chain. This evidence supports the hypothesis that there is a higher-affinity binding site on the neuronal membrane. Such a binding site could be a protein closely associated with the G_{1b} gangliosides. That the high-affinity binding site is not bound directly by the heavy chain of the neurotoxin could be explained by a conformational change in the heavy chain when bound first to the lower-affinity ganglioside which allows the heavy chain to recognize the high-affinity protein receptor.

The specific substrate of proteolytic cleavage by Lc of botulinum toxin type B and tetanus toxin was shown to be a specific isoform of synaptobrevin, synaptobrevin-2 (VAMP for vesicle associated membrane protein), an integral membrane protein of small synaptic vesicles (Schiavo et al., 1992b). VAMP is anchored to vesicles by a hydrophobic C-terminal tail structure while the remainder of the molecule remains exposed in the cytosol. Schiavo et al. (1992b) used highly purified synaptic vesicles from rat cerebral cortex to show that type B neurotoxin and tetanus toxin cleaved synaptobrevin-2 (VAMP) at Gln-76-Phe-77. Another VAMP, synaptobrevin-1 (VAMP-1), is virtually identical to VAMP-2 except that VAMP-1 has a valine residue substituted for the Gln-76 and it was resistant to cleavage by the endopeptidases neurotoxin B and tetanus toxin. The rate of cleavage of VAMP-2 correlated well with the inhibition of neurotransmitter release from *Aplysia* neurons indicating that the vesicle protein that was being cleaved was related to acetylcholine release (Poulain et al., 1989). Incubation of type B neurotoxin with VAMP-2 and a synthetic peptide that contained the cleavage site of VAMP-2 delayed the proteolysis

of VAMP-2. The next botulinal toxin serotype that was shown to cleave a synaptic vesicle protein was type F (Schiavo et al., 1993a). Type F neurotoxin was shown to cleave both isoforms of VAMP at a unique Gln-Lys peptide bond present in both VAMP isoforms. This site corresponds to Gln60-Lys61 of VAMP-1 and Gln58-Lys59 of VAMP-2.

The specific intracellular target of type D botulinal neurotoxin was shown to also be VAMPs. Type D neurotoxin cleaved both VAMP isoforms at a single site that corresponded to Lys61-Leu62 of VAMP-1 and Lys59-Leu60 of VAMP-2. These cleavage sites are one residue down from the target of type F neurotoxin (Schiavo et al., 1993b).

Type A and E botulinal neurotoxin were shown to have a different substrate than botulinal toxin types B, D, and F as well as tetanus toxin. Sollner et al. (1993) have shown that SNAP-25 (synaptosomal associated protein of 25 kDa) and VAMP are part of a multicomponent 20S protein complex proposed to facilitate vesicle docking and fusion. SNAP-25 was shown to be degraded by types A and E neurotoxin (Schiavo et al., 1993b; Schiavo et al., 1993c). The specific cleavage site was shown to be in the C-terminal region of SNAP-25. Using recombinant SNAP-25 the authors showed that the cleavage sites for A and E botulinal neurotoxins are separate and correspond to Gln197-Arg198 for type A neurotoxin and Arg180-Ile181 for type E neurotoxin.

The findings that botulinal toxin types B, D, F, and tetanus toxin have the same protein substrate which differs from that of toxin types A and E correlates well with the electrophysiological data that have placed the toxins in the same groups (Molgo et al., 1990).

V. Treatment of hyperactive muscle disorders with C. botulinum toxin.

History.

The use of type A C. botulinum toxin complex as a method of treating spastic muscle disorders was begun in the late 1970's by Drs. A. Scott and E. Schantz. The idea was to selectively paralyze hyperactive muscle groups by taking advantage of the mechanism of the toxin as an alternative to surgical procedures which involve mechanically severing nerves. Work began in earnest by the early 1980's using monkeys as models. After more than 20 years of collaboration between Scott and Schantz, crystalline type A toxin complex was licensed as an orphan drug in 1989 by the United States Food and Drug Administration. Approved treatments in patients 12 years of age and older include strabismus, hemifacial spasm, and blepharospasm (Schantz and Johnson, 1992). Since that time type A toxin has been used experimentally in the treatment of many other hyperactive muscle disorders. Some of these dystonias are summarized in Table 3. Dystonias are currently defined as "a syndrome of sustained muscle contractions, frequently causing twisting and repetitive movements of abnormal postures" (Schantz and Johnson, 1992). Treatment of spastic muscle disorders involves injection of nanogram quantities of the neurotoxin directly into the affected muscles. This creates a regional field of denervation and an overall reduction in muscle activity and pain (Borodic et al., 1991). Treatment duration varies with each indication but usually lasts for a period of several months.

Table 3. Uses of crystalline type A botulinal toxin in the treatment of spastic muscle disorders (adapted from Schantz and Johnson, 1992; Jankovic and Brin, 1991).

Condition	Symptom of disorder
Strabismus	Crossed eyes
Blepharospasm	Uncontrollable blinking
Hemifacial spasm	Facial twitching and spasms
Eyelid disorders	Inward turning of eyelid
Spasmodic torticollis	Abnormal movement of head and neck
Oromandibular and lingual dystonias	Sustained mouth closure or lingual muscle contractions
Focal dystonias of the hand	Writer's cramp, musician's cramp
Spasmodic dysphonia	Uncontrollable vocal spasms
Neurogenic bladder	Abnormal urinary control
Limb spasticity including cerebal palsy	Occurs following strokes; other neurologic disorders

Side effects of treatment with botulinum toxin.

The use of botulinum toxin in low dose applications (<20 LD50) has not been shown to cause adverse effects. Large dose applications such as those used in the treatment of spasmodic torticollis (>100 LD50) on a repetitive basis has caused a number of patients to develop neutralizing antibodies to the toxin (Jankovic and Brin, 1991; Scott, 1989). This is covered in more depth in chapter VI of this thesis. Other side effects from the use of botulinum toxin therapeutically include weakening and ptosis of nearby muscle groups due to the spread of the toxin from the point of injection. In treatment involving the muscles of the head and neck, one of the most prevalent adverse reactions is dysphagia or the inability to swallow. This has lead to upper airway obstruction in several patients (Borodic et al., 1991; Stell et al., 1988).

Properties of botulinum toxin for medical use.

In Figure 2, the SDS-PAGE electrophoretic pattern of the batch of type A toxin complex that was approved for use in humans (79-11) is shown. Lane 1 shows unreduced purified type A neurotoxin (molecular weight 145 kDa). Lane 2 (unreduced) shows the batch of toxin currently used medically (batch #79-11 produced at the UW-Madison, Food Research Institute in 1979 and stored at 4°C for 14 years). Lane 3 shows batch 79-11 reduced with 0.5% (w/v) dithiothreitol. Lane 4 shows molecular weight markers (from top to bottom in kDa) of 205, 116, 94, 66, 45 and 29. The molecular weights of the various toxin and nontoxic-complexing proteins present in lane 2 are as follows from the top of the gel (origin) in kDa: ca. 175, 147, 118, ca. 75, 50, 39, 35, 29, 22.8, 21.8, and 17. When 79-11 is treated with a disulfide reducing agent (lane 3), the Hc (93 kDa) and the Lc (52 kDa) of the toxin molecule are observed. Compared to other batches of toxin produced in our laboratory (see chapters III, IV, and V, this thesis), the batch 79-11 has deteriorated

substantially which may partly explain its relatively low specific toxicity (2-4 LD_{50}/ng, Botox® product insert) in the commercial product supplied to physicians.

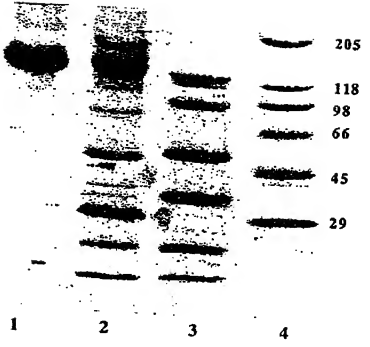

Figure 2. SDS-PAGE of purified type A neurotoxin and crystalline type A toxin complex batch 79-11 on a 8-25% acrylamide gradient gel. Lane 1, purified type A neurotoxin (unreduced); lane 2, batch 79-11 9 (unreduced); lane 3, batch 79-11 (reduced with 0.5 % w/v dithiothreitol); lane 4, molecular weight markers (rabbit myosin- 205 kDa, E. coli ß-galactosidase- 118 kDa, rabbit phosphorylase b- 98 kDa, bovine serum albumin- 66 kDa, ovalbumin- 45 kDa, carbonic anhydrase- 29 kDa) (Sigma Chemical Co., St. Louis, MO). All lanes contained 4-6 µg protein.

VI. Detection of Clostridium botulinum toxin.

Bioassay.

Detection of the toxin of Clostridium botulinum has been a concern since the time of van Ermengem in the late 1800's due to its presence as a contaminant in improperly handled foods. The classic method of detection is bioassay in animals. This method involves the injection of culture supernatant into a susceptible animal model and observing the animal for overt signs of botulism with an endpoint of death. The qualitative method of detection involves intraperitoneal injection of 0.1-0.5 ml of culture supernatant into 18-22 g white mice followed by observation for signs of botulism for the next four days. Animals that show signs such as labored breathing and a contraction of the oblique muscles of the abdomen within 2-24 h will usually die within the four day period. Serotype of culture supernatants that show the presence of botulinum toxin are determined by mixing samples of the toxic fluid with monovalent antisera that are specific for a single botulinal type. Confirmation of the presence of botulinal toxin is given when a single serotype of antiserum neutralizes the toxic effect. This presumes that there is only a single serotype of toxin present in the sample. A sample could contain two different serotypes of toxin that were produced by either a mixed population of botulinogenic organisms or by a single C. botulinum strain as in the case of Af producing strains. It is also possible that interfering substances from the sample cause death. In such cases, monovalent antisera will not protect the animal but dilution of the sample usually suffices to reduce this occurrence. Quantitation of botulinum toxin is done in terms of the amount of toxin needed to kill 50% of a population of test animals. This amount is termed 1 LD_{50} and is a statistical measurement based on the number of animals in each test group and the susceptibility of the test animals to the toxin. Quantitation is usually done with 5-10 animals /dilution and the results plotted semilogrithemically with % death on the vertical axis and dilution on the

horizontal axis. The point at which the line connecting the % death at each dilution crosses 50% is the dilution that contains 1 LD_{50}/injection volume (Schantz and Kautter, 1978). From this curve the number of LD_{50} in the original sample is calculated. This method is both expensive due to the animals used and time consuming as well. For these reasons other methods of detecting botulinum toxin have been devised.

An alternative method of determining the amount of active botulinum toxin in a sample is the intravenous time-to-death method of Boroff and Fleck (1966). This method involves injection of a sample into the tail vein of an immobilized mouse. The time-to-death is noted and converted to the number of intraperitoneal LD_{50} using a standard curve prepared using the dilution to extinction method of Schantz and Kautter (1978). The method is fast and relatively accurate (±20%). Drawbacks to this method are the preparation of the standard curve and the need for a higher level of technical expertise. The method is also applicable only when the amount of toxin in the sample falls within the linear portion of the standard curve (1×10^6- 5×10^3 LD_{50}/ml of type A toxin).

Serologic methods.

Antibodies raise against detoxified botulinum toxin specific to a single serotype have been used in a number of ways to detect and quantitate the amount of toxin in given sample. A few of these serologic methods are presented below.

Passive hemagglutination/reversed-passive hemagglutination.

Passive hemagglutination involves formalin treated sheep red blood cells that are conjugated to toxin molecules or fragments of toxin molecules while reversed-passive hemagglutination uses antibodies specific to individual toxin serotypes conjugated to blood cells (Gordon et al., 1958; Johnson et al., 1966; Sakaguchi et al., 1974). The assay is done by adding dilutions of the corresponding toxin sample or antitoxin sample to the

sensitized sheep red blood cells in a microtiter plate and examining the plate for agglutination of the blood cells. It has been reported that the sensitivity of the reversed-passive hemagglutination assay using polyclonal IgG antibodies purified by affinity chromatography and conjugated to sheep red blood cells was as low as 8-10 LD_{50}/ml for types A and B toxins (Sakaguchi et al., 1974). The method of reversed-passive hemagglutination suffers from some of the following problems that are not uncommon to other antibody based botulinum toxin assays: 1) the assay does not distinguish between biologically active toxin and inactive toxin; 2) cross-reaction is seen between different serotypes presumably due to the quality of the coupled antibodies; and 3) hemagglutination is a characteristic of some of the botulinal toxin complex proteins, specifically A, B, and F, and may be a characteristic of other components of the sample being tested.

Immunodiffusion.

Immunodiffusion has been used to detect botulinal toxins in a manner analogous to the Ouchterlony assay (Crowle, 1958; Wadsworth, 1957) whereby toxin and antitoxin specific for a given serotype of toxin are allowed to diffuse toward each other through agar or agarose. At the point at which the two preparations converge, a visible line of immunoprecipitate is formed (Vermilyea et al., 1968). There are variations on the technique using microscope slides (Vermilyea et al., 1968) as well as capillary pipets partially filled with antitoxin mixed with agar (Mestrandrea, 1974). This method suffers from a lack of sensitivity (on the order of 300-500 LD50/ml) as well as not being able to distinguish between active and inactive toxin.

Radioimmunoassays.

Radioimmunoassays based on the ability of an antibody to recognize a specific serotype of botulinum toxin were first used in 1973 (Boroff and Shu-Chen, 1973). The method commonly uses ^{125}I labeled toxin and antibodies specific for a given serotype. Unlabeled toxin present in a sample competitively binds the fixed amount of antiserum present. Hence, the more toxin present, the lower the radioactivity recovered bound to antibodies. One difficulty encountered is the fact that inactive toxin may be present and give too high an estimation of the amount of toxin present (Betley and Sugiyama, 1979). This noncorrelation between toxicity and antigenicity is the major drawback in all serological quantitations of botulinum toxin. Another obvious drawback to radioimmunoassays is the use of radioactive isotopes and the difficulties encountered in handling and disposing of them.

Enzyme linked immunosorbent assays.

ELISAs have been used for the detection of botulinum toxin since the early work of Notermans et al. (1978) in which a tube ELISA was described. The various ELISA methods use an antibody or series of antibodies specific for a given toxin serotype to capture and label the toxin. Typically in a double-sandwich type ELISA a tube or microtiter plate is coated with a capture antibody such as horse anti-botulinum type A. The sample is bound by the capture antibody and the toxin then labeled with another antibody specific to the toxin but from a different source than the first antibody. A third antibody labeled with a chromogenic enzyme is added that is specific for the second antibody, and color developed by addition of substrates for the chromogenic enzyme.

The major difficulty encountered in using the ELISA method for detection of botulinum toxin is the cross-reaction seen between different serotypes of toxin (Goodnough et al., 1993; Notermans et al., 1978). This cross-reaction is due to the

presence of antibodies that react to epitopes common to more than one serotype of toxin or to epitopes common to non-toxic complex proteins that contaminated the toxoid (Somers and DasGupta, 1991). There are numerous regions of homology that are conserved between the individual serotypes of toxin (Niemann, 1991; Whelan et al., 1992; Tsuzuki et al., 1988). The solution to the first difficulty, namely, contamination of the toxin antigen with non-toxic proteins from the complex can not be avoided entirely. However, neurotoxin preparations for use in antibody production can be made that are purified to the extent allowed by modern chromatography. The difficulty arising from the presence of a subpopulation of antibodies reacting to conserved regions of different toxin serotypes in a polyclonal pool has been addressed by the use of monoclonal antibodies (Kozaki et al., 1986; Gibson et al., 1988; Ferreira et al., 1990). An additional difficulty in the use of ELISAs for detection of botulinum toxin is the fact that the detection levels for most of the assays are in the range of 20-10,000 LD_{50}/ml (Notermans et al., 1978; Modi et al., 1987; Goodnough et al., 1993; Gibson et al., 1988). A method using snake venom as part of an amplification system to detect botulinum toxins has recently been published that claims to be as sensitive as the mouse bioassay detecting ca. 10 pg of neurotoxin (Doelgast et al., 1993).

Others.

The polymerase chain reaction (Saiki et al., 1988) has been successfully used for detection of Clostridium botulinum in culture media (Szabo et al., 1992). This method used a set of primers specific for the type B toxin gene. The method detected as few as 100 fg of the target DNA (ca. 35 cells). The major drawback to this method is the fact that it can not distinguish between the organism and the etiologic agent, the toxin. The organism may be present without producing toxin as in the case of the dormant endospores. Its sensitivity is likely to be less in foods.

VII. Lyophilization.

Lyophilization is the method preferred to stabilize toxin for commercial supply to physicians. Freeze-drying or lyophilization, is a process in which the solvent is first frozen and then removed by sublimation in a vacuum environment (Pikal, 1990). The solvent is usually water and is frozen in commercial freeze-dryers at a temperature of ca. -40°C. When the frozen product has solidified, the drying chamber is evacuated and the shelf temperature raised slowly to initiate the sublimation of the ice crystals. The water that is sublimed is removed from the drying chamber and collected on low temperature condensers (-60°C). The first stage of drying is termed primary drying and typically removes 50-80% of the water present. The water remaining in the product after primary drying is removed during secondary drying. Secondary drying is typically done at elevated shelf temperatures (0-25°C) in order to remove the water remaining in the amorphous solid.

Freezing the water in a solution concentrates solutes in the solution to the point where many of them crystallize. Those that do not crystallize are transformed into a rigid glass when the system is brought below the glass transition temperature of the amorphous phase (Pikal, 1990). The glass transition temperature (Tg') is the point at which water in the mixture ceases to form ice crystals during freezing at least on a realistic time scale. This unfrozen water is characterized by the Tg' and its water content. The properties of the amorphous mixture change at the Tg'. Above the Tg', the water that is present in the amorphous mixture is free to diffuse and the viscosity of the material drops. The water that is present is free to participate in various chemical reactions such as deamidation, aggregation, and peptide bond hydrolysis which are all dependent on free water. Below the Tg', the mixture of water and glass is more rigid and diffusion rates are extremely slow on the order of μm/year (Franks, 1990).

Crystallization of the water occurs after supercooling of the solution to 10-15°C below the equilibrium freezing temperature. This temperature varies depending on the

solutes, the shelf temperature, and on the presence of particulate material to serve as ice nucleation sites. The degree of supercooling determines the size of the crystals and therefore is intimately associated with the structure of the freeze-dried product. The higher the supercooling, the smaller the ice crystals and the smaller the pores in the amorphous solute matrix. Smaller pores have a larger surface area than larger pores and so facilitate both primary and secondary drying.

Primary drying is a process of mass transfer from the frozen vials to the condenser of the freeze-drier. For sublimation of each gram of ice, ca. 1,000 liters of water vapor passes through the partially dried cake of material in each vial (Pikal, 1990). The rate of sublimation is dependent on the difference in pressure between the water vapor in the frozen product and the condenser of the chamber. The resistance to collection of the water vapor is dependent on vial size and shape, stopper configuration, and resistance across the partially dried cake. The first two can easily be controlled while the resistance to water vapor escape through the dried product can be controlled by the freezing process and the degree of supercooling of the solution. Temperature of the material is slowly raised during primary drying which also contributes to the rate of sublimation. Primary drying ends when all of the ice in the vials has been removed. The partial pressure of water vapor present in the drying chamber is nearly equal to the total during primary drying but drops sharply at the end of primary drying.

Secondary drying begins after the ice in the vials has been removed and continues until the final percent moisture levels have been reached. For non-protein systems this level may be less than 1% while protein products typically contain up to 4% water. This difference is primarily due to the differences in formulation with protein products using higher levels of cryoprotectants such as carbohydrates which have high levels of water of hydration (Franks, 1990).

Excipients used in freeze-drying.

Compounds added to a solution to be lyophilized in addition to the active components are termed excipients. Excipients may consist of bulking agents to prevent "blowout" of the product. Blowout can occur when solutions with very low % solids are freeze-dried. The water vapor from sublimation can carry pieces of the freeze-dried material out of the vial in such cases. Buffers are frequently used which contribute to the overall % solids of the solution. Some products have salts such as sodium chloride added to yield isotonic solutions when reconstituted and still others have compounds such as arginine added to increase the solubility of the active component. Protein pharmaceuticals typically have a lyoprotectant added to stabilize the active compound during the freeze-drying cycle and subsequent shelf-life. Some of the compounds used for stabilization of protein pharmaceuticals include dextran, polyvinylpyrrolidone, polyethylene glycol, ficoll, gelatin, serum albumin, hydroxyethyl starch, trehalose, sucrose, lactose, arginine, glycine, and mannitol. The addition of inert proteins to a formulation containing pharmaceutical proteins of interest has been shown to stabilize the active moiety in solution and during lyophilization (Schantz, 1964; Schantz and Kautter, 1978; Goodnough and Johnson, 1992). However, the material usually has a relatively low shelf-stable temperature. In the case of botulinum toxin, the storage temperature for the commercial product is <-10°C. This is presumably due to the low Tg' of the freeze-dried material. This issue is further addressed in Chapter IV of this thesis. Addition of polyhydroxylated compounds such as carbohydrates has been shown to provide a more shelf stable freeze-dried product than the corresponding product lacking the carbohydrate (Colaco et al., 1992; Mouradain et al., 1984; Roser, 1991; Franks et al., 1991). The mechanism by which carbohydrates such as trehalose stabilize protein molecules is unknown. One possibility is that by raising the glass transition temperature of the freeze-dried material, the storage temperature of the material is correspondingly elevated. It is also possible that the carbohydrate is somehow

replacing the water molecules that are involved in maintaining the tertiary structure of the molecule during lyophilization. Such an idea is termed the water replacement hypothesis and was proposed by Saenger (1989) and Otting et al. (1991). Others (Levine and Slade, 1988; Franks et al., 1991) have argued that the tendency for carbohydrates to form glassy states does not allow the molecular movement needed for the protein of interest to undergo degradative reactions.

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CHAPTER III

Colony immunoblotting of Clostridium botulinum types A, B, and E

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Abstract

Neurotoxin produced by colonies of Clostridium botulinum types A, B, E and toxigenic Clostridium butyricum bound to nitrocellulose was detected by an enzyme-linked immunoassay procedure. The procedure used serotype specific rabbit IgG as the primary antibody and goat antirabbit IgG antiserum labelled with alkaline phosphatase as the marker to visualize immobilized neurotoxin from individual colonies. The method differentiated the cblonies based on serotype of neurotoxin produced and by the amount of neurotoxin produced by individual colonies on the same agar plate. Specificity of primary antibodies used was improved by adsorption of cross-reacting heterologous antibodies.

Introduction

The technique of using specific antibodies for the detection of immobilized proteins of interest has been described extensively since 1979 (Towbin, 1979). The more common techniques involve transfer of proteins from polyacrylamide-gels to a solid support such as nitrocellulose using an electrical current (Bittner et. al., 1980). The transferred proteins are then probed with antibodies specific for the protein of interest. In contrast to this electroblotting technique, we have been using for sometime in our laboratory a colony-blotting procedure for the detection, differentiation, and estimation of the varying toxin titers of the individual Clostridium botulinum colonies of types A, B, and E as well as two toxigenic Clostridium butyricum strains. The method uses alkaline phosphatase as the enzyme marker (Mason, 1978) and nitrocellulose as the solid support.

The potential uses of this technique include the screening of various ingredients in food products to determine the C. botulinum spore load prior to formulation as well as the efficacy of the subsequent heat-treatment of food products. Temperature abuse as well as adequacy of the total anti-microbial system could be monitored by simply using selective media followed by blotting. The follow-up procedure must include a more sensitive toxin assay system such as the mouse bioassay. The colony-blotting technique could potentially find use in the screening of products which do not receive a heat treatment to determine if there are C. botulinum spores present and at what levels. The colony blotting assay has found use in our laboratory as a convenient method of screening large numbers of potential mutants for toxin production.

Materials and Methods

Organisms.

Clostridium botulinum type A strains were Hall A, 73A, 90A, 109A, and 62A. C. botulinum type B strains were Okra B, Lammana B, 32B, 7949B, 113B, 213B, and 169B. Clostridium sporogenes strains 4411 and PA 3679 were used as the negative controls in the type A and B blotting tests. Type E C. botulinum strains were Alaska E, 5545E, and Iwanii E. All C. botulinum and C. sporogenes strains were from the Food Research Institute culture collection. Clostridium butyricum strains 5839 and 5521 produce a neurotoxin very similar to the botulinum type E toxin molecule (Gimenez and Sugiyama, 1988a). These toxigenic strains were obtained from Dr. C. Hatheway, Centers for Disease Control, Atlanta, GE. Non-toxigenic C. butyricum strains 19398 from the American Type Culture Collection and 1024 from the Food Research Institute collection were used as negative controls in the type E blotting procedure.

Culture media.

Colonies for the blotting tests were usually grown on TPGY agar (5% trypticase peptone, BBL; 0.5% bacto-peptone, Difco; 0.4% glucose; 0.2% cysteine-HCl; 0.1% yeast extract, Difco; 2.0% bacto-agar, Difco; pH 7.4) which supports growth of all test strains. In some cases, the three antibiotics of CBI agar (C. botulinum isolation medium (cycolserine, 250 mg/l, sulfamethoxazole, 75 mg/l, trimethoprim, 4 mg/l) (Dezfulian et al., 1981) were added to TPGY to make the medium selective for C. botulinum types A and B as well as some strains of type E.

C. butyricum colonies were sometimes grown on a minimal medium consisting of 1.0% glucose, 0.1% $(NH_4)_2SO_4$, 4.0% salts solution (2mM $CaCl_2$, 1.7mM $MgSO_4$, 5.7mM K_2HPO_4, 7.4mM KH_2PO_4, 120mM $NaHCO_3$, 34mM NaCl), 0.4% resazurin

solution (250µg/ml), 0.4% biotin (12.5µg/ml), 0.1% thiamine-HCl (1mg/ml), 0.05% cysteine-HCl, and 0.5% $NaHCO_3$). This GMBT medium is a modification of the GMB medium described in the VPI Anaerobe Manual, 4th edition (Holdeman, Cato, and Moore, eds.).

Antiserum.

Antitoxins for type A, B, and E botulinum toxin serotypes were raised in rabbits. Type A toxin was purified by the method of Tse et al. (1982), type B toxin by the method of DasGutpa and Sugiyama (1976), and type E toxin by the method of Gimenez and Sugiyama (1987). These preparations were considered to be pure toxin samples since they showed only the approximate 150 kDa protein when electrophoresed in polyacrylamide gels without being treated with a disulfide reducing agent. When disulfide bonds were reduced with mercaptoethanol (1%v/v) or dithiothreitol (0.5% w/v), the preparations showed the characteristic ca. 100 kDa heavy chain and the ca. 50 kDa light chain.

The toxins were converted to toxoids by dialysis against 50 mM sodium phosphate buffer, pH 8.0 with 0.4% formalin for 14 days at 30°C. Most of the unreacted formalin was removed by dialyzing the toxoids against 65 mM sodium phosphate buffered saline (PBS), pH 7.4. The toxoids were homogenized in equal volumes of complete Freund's adjuvant (Difco Laboratories, Detroit, MI). The mixture was injected subcutaneously into rabbits in volumes containing 200-300 µg of toxoid. Each rabbit was then given a SC injection of ca. 100 µg of native toxin (ca. 10^7 mouse 50% lethal doses) on days 37, 38, 39, 44, 45, 46, 51, 52, and 53. The rabbit was then bled by cardiac puncture on about day 60.

The serum (ca. 50 ml/rabbit) was dialyzed against 20mM Tris-HCl, pH 8.5 and applied to a Sepharose CL4B-Protein A column (1.6 cm x 22 cm) equilibrated in the same buffer at a rate of 30 ml/hr. After loading the crude serum, the column was washed with 5

column volumes of the loading buffer folowed by a similar volume of 50 mM citrate/50 mM NaCl, pH 7.0. IgG bound to Protein A was eluted in a single protein peak when the buffer was changed to 50 mM citrate/50 mM NaCl, pH 3.0. The IgG fractions were pooled as soon as practical and the pH adjusted to ca. 7.0 with 1.0 M Tris-HCl, pH 9.0. The antitoxin was then dialyzed against 65 mM PBS, pH 7.4, and stored at 4ºC in a final concentration of 25% glycerol.

Adsorption.

The type A, B, and E antitoxins were type specific in toxin neutralization tests so that type A antitoxin did not neutralize type B or E toxin, type B antitoxin did not neutralize type A or E toxin, while type E antitoxin did not neutralize type A or B toxin. However, some in vitro serological procedures with the type A and B antitoxin types did cross-react to some extent with the heterologous toxin. In the past, these cross-reactions have made the distinction between toxin serotypes difficult and unreliable using the immunoblotting procedure. This cross-reaction is most likely due to the presence of small amounts of non-toxic complex proteins including hemagglutinating proteins in the antigenic preparations. Some of these non-toxic proteins have epitopes common to all of the toxin serotypes (Somers and DasGupta, 1992). In order to minimize these cross-reactions an adsorption procedure was developed. The procedure involves the addition of whole cells and concentrated crude toxin preparations of the heterologous toxin types to the IgG antiserum preparations of type A and B. The type E antiserum preparation did not cross-react to the extent of the A and B antiserum and was not adsorbed.

The strains used as adsorbants for type A antitoxin were C. botulinum strains 113B, 213B, and Okra B. Type B antitoxin was adsorbed with C. sporogenes strain 4411, and C. botulinum Hall A. Toxin concentrates were the precipitate formed when 450 ml of a 4 day TPGY culture was adjusted to pH 3.5 with 1N HCl. This was a modification

of the method of Tse, et. al. (1982). The precipitate was collected by centrifugation and suspended in ca. 5 ml of 65mM PBS, pH 7.4. Cells for use in the adsorption procedure were grown seperately for 24 hr at 37°C in 50ml of TPGY broth, pH 7.4, collected by centrifugation, and washed three times with sterile 0.85% physiological saline. The separate strains for the respective antitoxins were combined and resuspended in ca. 5 ml of saline.

After combining crude toxin/whole cell preparations, a known number of international units (1 IU for types A and B = 10,000 mouse LD_{50}; 1 IU for type E =1,000 mouse LD_{50}) of heterologous antiserum was added to ca. one-third of the total volume of adsorbant and allowed to react at room temperature for 30 min to 1 h. The mixture was then centrifuged at 12,100 x g for 20 min. The supernatant was readsorbed twice in the same fashion. After the final adsorption, the antiserum was dialyzed against 20mM Tris-HCl, pH 8.5, and reapplied to the Protein-A column. The column was then extensively washed with the loading buffer (30-50 column volumes) to remove the heterologous toxin present. The adsorbed IgG fractions were then eluted as before with 50 mM citrate/50 mM NaCl, pH 3.0. The resulting antiserum preparation had ca. 10-100X less cross-reactivity than the parent antiserum. Each antiserum preparation was then retitrated against its homologous toxin type yielding values from 90 to 400 IU/ml for types A and B and 340 IU/ml for type E antiserum.

Colony blotting procedure.

The procedure used here is a direct immunostaining procedure of immobilized proteins. The complete immunosandwich is depicted schematically in Figure 1. Plates with surface inoculated colonies were grown anaerobically $\geq$48 h. Colonies were reasonably well isolated with ca. 100 colonies/plate maximum to ensure no overlap of antigen as toxin diffuses away from the colonies through the agar medium.

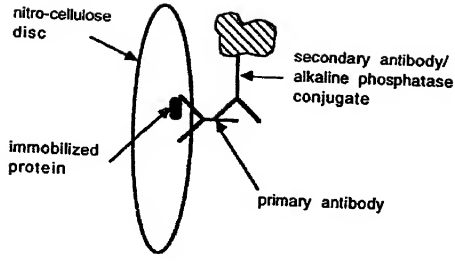


Figure 1. Schematic representation of immunoblotting of Clostridium botulinum colonies on nitro-cellulose discs.

The immobile phase used to bind the neurotoxins was nitrocellulose (NC) from Bio-Rad Laboratories, Inc., Richmond, CA. The NC came pre-cut into 82.5 mm diameter discs which were applied directly to the agar plates. The first NC disc was placed on top of the colonies by dragging the leading edge of the disc with forceps over the rim of the plate until the trailing edge dropped onto the plate. The NC became wetted slowly from the trailing edge toward the leading edge. This method of application ensured no air bubbles were trapped under the NC prohibiting contact with the plate and the colonies. The plate with the NC adhering to it was then inverted and held at room temperature for 2 h. The NC was removed from the plate by peeling it off in the reverse order of application. This NC disc could then be overlaid briefly onto a fresh plate and a replicate of the master plate made before the disc was blotted. This step required well dispersed colonies. A second disc could be overlaid onto the master plate at this time and the NC/plate incubated under the same conditions.

After removal from the master or duplicate plate, the colonies which adhered to the NC were removed with a gentle stream of distilled water. The NC was then placed into a blocking solution to completely saturate the unbound protein binding sites on the disc. The blocking solution consisted of 5% skim milk powder in TBS (10 mM Tris-HCl, 0.9% NaCl, 0.1% bovine serum albumin, pH 7.4) (Sigma Chemical Co., St. Louis, MO). Alternatively, 5% bovine serum albumin in TBS was substituted. These incubations were done in petri dishes with gentle agitation for 1 h at room temperature.

After 1 h incubation, the blocking solution was replaced with primary antiserum diluted with TBS + 1% bovine serum albumin, pH 7.4. Working concentrations of primary antiserum varied from one antiserum lot to the next. Optimum dilutions ranged from 0.8 IU/ml for some of the type A and B antisera to 10 IU/ml for the type E antiserum. Total volume used in the primary incubation step was 10.0 ml for a total of ca. 8-10 IU for

the type A and B blots and 100 IU for the type E blots. This incubation was carried out at room temperature for 1 h with agitation as before.

At the end of the primary antiserum incubation, the solution was decanted and the blot washed with TBS + 0.09% Tween 20, pH 7.4, to remove excess unbound antibodies. Three 75 ml washes were carried out in a large, flat-bottomed, plastic, storage container (ca. volume 1500 ml) with agitation for 10 min each. At the end of the third wash, a brief (2 min) rinse was done with TBS to remove excess detergent.

NC discs were transferred to clean petri dishes containing 10.0 ml of secondary antiserum (goat anti-rabbit IgG conjugated with alkaline phosphatase, Boerhinger-Mannheim, Indianapolis, IN) diluted in TBS + 1% bovine serum albumin, pH 7.4, to a final concentration of 1:2000. The blots were incubated with agitation at room temperature for 1 h and then washed as before in TBS + 0.05% Tween 20 and 0.05% SDS, pH 7.4, for 10 min each. A brief rinse (2 min) with TBS was done to remove detergent which might interfere with enzymatic activity. After washing, the enzyme substrates were added in a 100 ml volume of 1 M Tris-HCl, pH 9.5. The substrates used were 5.6 mM 5-bromo-4-chloro-3 indolylphosphate (BCIP) plus 4.8 mM nitroblue tetrazolium (NBT) (both from Sigma). NBT was first made soluble in ca. 10 ml of boiling 1 M Tris-HCl, pH 9.5, and then added to the remaining 90 ml of 1 M Tris-HCl, pH 9.5. BCIP was dissolved in 100 µl of dimethylsulfoxide and then added to the 1 M Tris-HCl; pH 9.5. After mixing, the substrates were added to the blots in the wash container and the purple color allowed to develop until the background began to appear (usually within 2-5 min).

After full color development, the reaction was stopped by thoroughly rinsing the blots in distilled water and allowing them to air dry in the dark. The colored complex is light sensitive and will gradually fade upon exposure to sunlight.

The entire process of colony blotting may be stopped prior to any of the above steps by simply holding the blot in TBS. Blotting may then be resumed the following day with

no change in sensitivity. Alternatively, prior to development, the blots may be rinsed briefly in TBS and then forzen at -20°C for weeks to months without any change in reactivity.

SDS-PAGE.

Polyacrylamide gel electrophoresis was done using the Bio-Rad Protean II system (Bio-Rad Laboratories, Richmond, CA). Linear 12.5% acrylamide gels were run at 20 mA constant current at room temperature according to the discontinuous system of Laemmli (1970).

Electrotransfer and immunoblotting of SDS-PAGE gels.

SDS-PAGE gels of C. botulinum toxin samples were transferred to polyvinylidene difluoride membranes (PVDF) (Millipore, Corp., Bedford, MA) using the Bio-Rad Trans-blot Electrophoretic Transfer Cell. Transfers were made overnight at room temperature and 30 mA constant current in 10mM CAPS (3-(cyclohexylamino)propanesulfonic acid) (Sigma), 5% methanol, pH 10, following the procedure of Matsudaira (1987). Transferred proteins were visualized using the immunoblotting procedure described for colony blots. All incubations and washes were done in large flat-bottom (1500 ml) containers.

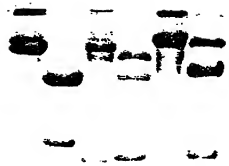
Results

In order to determine whether the adsorption procedure for removal of cross-reacting antibodies from the type A and B antisera was effective, known amounts of crude culture of both toxin types in 10-25 µl volumes were spotted side-by-side onto NC and the immunoblotting procedure performed as described for colony blotting using the antisera in question. The developed reactions showed that the reduction in cross-reaction was typically 10-100 fold. These dot-blots were also used to determine the limits of detection. Dilutions of each serotype of toxin were spotted onto the nitro-cellulose followed by the colony-blotting procedure. The limit of detection for the type A and B antiserum batches was in the range of 10-25 LD_{50}. Assuming a specific activity of 30 LD_{50}/ng for type A toxin complex, this is equivalent to 300-800 pg of toxin complex or 100-267 pg of purified type A neurotoxin.

Electrotransfer of type A toxin, type A toxin complex, and type B neurotoxin from a SDS-PAGE gel was done to establish whether type A antibodies reacted with type B neurotoxin. Figure 2 shows a 12% polyacrylamide gel which has been stained with 0.1% Coomassie brilliant blue R250 (Sigma) in 7.5% acetic acid and 25% methanol. Lanes 1 (unreduced) and 2 (reduced with 0.5% w/v dithiothreitol) contain purified type A toxin, lanes 3 (unreduced) and 4 (reduced) contain type A complex, and lanes 5 (unreduced) and 6 (reduced) contain purified type B toxin. Each lane was loaded with 10-15 µg of protein.

Figure 3 shows the corresponding immunoblot from the gel depicted in Figure 2; it was developed after reaction with adsorbed type A IgG. Lanes are numbered in the reverse order of those in Figure 2. Purified type B toxin (lanes 1 and 2) is unreactive at this protein concentration while type A complex (lanes 3 and 4) and purified type A toxin (lanes 5 and 6) show reaction with the unreduced and the reduced toxin but not with the non-toxic proteins associated with the type A toxin complex. There is a faint signal in the

region of ca. 30kDa in both the reduced type A toxin samples which could be indicative of a breakdown fragment of the toxin molecule.



1 2 3 4 5 6

Figure 2. SDS-PAGE of Clostridium botulinum toxins. Lane 1, type A neurotoxin; lane 2, type A toxin neurotoxin (reduced with 0.5% w/v dithiothreitol); lane 3, type A toxin complex; lane 4, type A toxin complex (reduced), lane 5, type B neurotoxin; lane 6, type B neurotoxin (reduced). Each lane was loaded with 10-15 µg of protein.

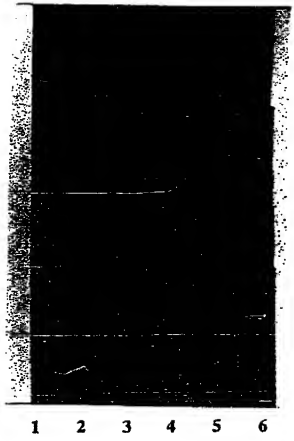


Figure 3. Immunoblot of proteins after electrophoretic transfer from the SDS-PAGE gel shown in Figure 2 using adsorbed type A IgG as primary antibody. Lane 1 (reduced) and lane 2 (unreduced) contain purified type B toxin, lane 3 (reduced) and lane 4 (unreduced) contain type A toxin complex, lane 5 (reduced) and lane 6 (unreduced) contain purified type A toxin. Each lane of the original gel was loaded with 10-15 μg of protein prior to electrophoresis and electrophoretic transfer.

We found that of the three toxin types there were some strains which cross-reacted to a much greater extent than others. Hall A and 62A cross-reacted with the unadsorbed type B antiserum as did Okra B and 113B with the type A antiserum. The cross-reaction was not completely eliminated after adsorption as determined with the dot-blot procedure but was substantially reduced and did not interfere with the colony blotting procedure. Colonies of C. sporogenes 4411, PA 3679, and a C. tetani strain did not react with any of the adsorbed antisera.

A typical type A colony reaction is shown in Figure 4. It shows that the toxin which has diffused away from the colony is still bound to the NC and was detected. Because of this it is possible to overlay a second disc and develop it under different conditions using a different primary antiserum. This technique is shown in Figures 5, 6, and 7. Figure five shows the original plate which contained a mixed population of predominantly type A toxin producing colonies. The arrowheads indicate three colonies which produced type B toxin. The first disc overlaid on the plate was developed in type A specific antiserum (Figure 6) and the second disc in type B specific antiserum (Figure 7).

Figure 4. Typical type A *C. botulinum* toxin producing colony reaction on nitrocellulose using colony immunoblotting procedure.

Figure 5. TPGY agar plate containing a mixed population of C. botulium type A and C. botulinum type B. The arrowheads indicate the type B colonies (from Goodnough et al., 1993).

Figure 6. Colony immunoblot of mixed population of C. botulinum types A and B toxin producing colonies using antitype A IgG as primary antibody (from Goodnough et al., 1993).

Figure 7. Colony immunoblot of mixed population of C. botulinum types A and B toxin producing colonies using antitype B IgG as primary antibody (from Goodnough et al., 1993).

Diffusion of toxin through the culture medium is also demonstrated in Figures 8 and 9. Figure 8 shows the size of the co-cultured C. butyricum strains 5839 (toxigenic) and 19398 (non-toxigenic) as grown on the minimal medium GMBT and the size of the reaction zone from the toxigenic strain (Figure 9). In all cases the plate and the blot are mirror images of each other. In Figure 9, the "negative" zones of non-toxigenic C. butyricum 19398 can be seen as lighter spots in the much darker "positive" zones of the toxigenic strain 5839. Individual colonies from the original plates or from a duplicate plate can then be reisolated.

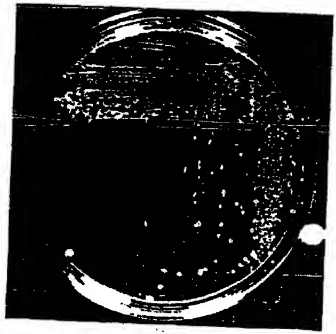


Figure 8. Mixed colonies of toxigenic C. butyricum (strain 5839) and nontoxigenic C. butyricum (strain 19398) on GMBT minimal medium.

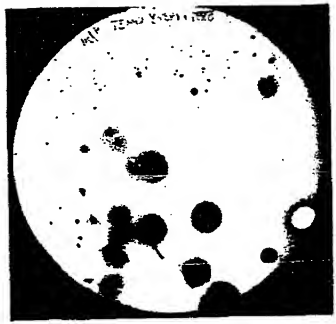


Figure 9. Colony immunoblot using antitype E IgG as primary antibody. A mixed population of toxigenic C. butyricum (strain 5839) and nontoxigenic C. butyricum (strain 19398) was grown on GMBT minimal medium. The arrow indicates the position of a nontoxigenic C. butyricum colony in the darker background of toxin which has diffused from a nearby toxingenic C. butyricum colony.

Another use of this blotting procedure is that it can distinguish between high-titer and low-titer colonies on the same agar plate. Figure 10 shows a blot of colonies of C. botulinum 7949 B. The different intensity reactions are indicative of the toxin titers of the colonies. When isolated individually and grown in TPGY broth culture the colony giving the lighter reaction (indicated by the arrow) produced 10 fold less toxin/ml than the colony which gave the darker reaction.

Figure 10. Colony immunoblot of C. botulinum type B (strain 7949) toxin producing colonies. The lighter reacting colony indicated by the arrow produced ca. ten-fold less toxin when isolated, grown in broth culture, and tested in the mouse bioassay (from Goodnough et al., 1993).

Discussion

The sensitivity of the blotting procedure in our hands is on the order of 10-25 LD_{50}/spot. Assuming a relative toxicity for type A toxin complex of 3×10^7 LD_{50}/mg (Schantz, 1964), this detection limit is on the order of 300-800 picograms. This limit compares favorably with other enzyme-linked immunosorbent assays (Lyeryl, 1983; Kozaki, 1979; Laughon, 1984; Notermans, 1978). The limit of detection is critically dependent on the quality of the primary antiserum.

Cross-reaction of the different serological types of antisera could be due to the presence of antibodies to common non-toxic proteins associated with the different toxin serotypes or to similar epitopes being present on the different serotypes of toxin. These non-toxic proteins are conserved throughout the range of botulinal serotypes (Somers and DasGupta, 1991) and any trace of carry over from the toxin purification to the antigenic preparation will generate cross-reactions in the blotting procedure. It is possible there are individual epitopes which are common to more than one serotype as well. The use of monoclonal antibodies may be of help to avoid these cross-reactions.

The technique of colony-blotting is an alternative method to the mouse assay for the detection of Clostridium botulinum neurotoxins. The traditional method of picking individual colonies followed by growth in broth medium and toxin testing in mice is time consuming and expensive. The standard mouse bioassay is still the method of choice when detection of very low quantities of toxin ($\leq$10 LD_{50}) is necessary. However, for some laboratories without the facilities to properly house animals for toxin testing, this method may be useful. An additional use of the colony blotting procedure is in detection of tox$^-$ mutants in a population of mutagenized C. botulinum. This has been used in our laboratory in conjunction with transposon mutagenesis (Lin and Johnson, 1991) of C. botulinum type A. It may be possible to detect so called leaky mutants in regulation studies

of toxin formation using the colony blotting approach which is much easier than screening individual colonies for toxin titer.

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CHAPTER IV

Characterization of type A Clostridium botulinum toxin complex during purification

Abstract

Purified type A Clostridium botulinum neurotoxin is used for the treatment of spastic muscle disorders due to the fact that it causes a flaccid paralysis in specific muscle groups when very dilute solutions of the toxin are injected. The toxin for medical use is produced by the Hall A strain as part of a complex of at least six other proteins and is purified by a series of precipitations and crystallizations. In this study the toxin complex was examined at each step in the purification for total toxicity, percent solids, optical density at 260 and 278 nm, specific toxicity, and for the presence of ribonucleic acids. The results show that the procedure is variable and dependent to a large degree on the individual components making up the growth medium indicating that nutrition and regulation affect toxin quality. RNA associated with the purified toxin complex was found at a level of 0.3% and electrophoretic analysis indicated that particular species of RNA were not present in the complex.

Introduction

Clostridium botulinum is a heterologous group of gram positive, obligately anaerobic, endospore forming rod-shaped organisms that have in common the production of a characterisitic proteinaceous neurotoxin. There are currently seven known serotypes of neurotoxins (A, B, C_1, D, E, F, and G). These neurotoxins are most commonly known as being the cause of the food borne intoxication botulism (Sugiyama and Sofos, 1988). These proteins are among the most potent neurotoxins known with lethal doses on the order of 1 ng/kg for most mammals including humans (Schantz and Johnson, 1992). The mode of action of the neurotoxin is such that it binds to the presynaptic junction of motor neurons and prevents the release of the neurotransmitter acetylcholine. This lack of signal to the particular muscle group innervated by the neuron causes a flaccid paralysis. Death can result from the toxins action on muscles of the respiratory system including the diaphragm and the intercostals.

Type A toxin when diluted to very low concentrations (ng/ml) is currently used therapeutically for the treatment of spastic muscle disorders (Jankovic and Brin, 1991; Savino and Maus, 1991). Target muscle groups are injected directly causing a regional paralysis and denervation (Borodic et al., 1991). A single batch of purified toxin was approved by the U. S. Food and Drug Administration for treatment of disorders such as strabismus, blepharospasm, and hemifacial spasm in December of 1989 (Schantz and Johnson, 1992). This particular batch was produced by the Hall A strain of C. botulinum in a simplified culture medium consisting of casein hydrolysate, yeast extract, and glucose.

Production of type A toxin by the Hall strain in a simplified growth medium was first elucidated by Lewis and Hill (1947). The purpose of their research was to establish that the organism could produce toxin titers in the range of 5×10^5-1×10^6 mouse lethal

doses/ml of culture in such a medium. This research was pursued due to difficulties in World War II in obtaining the necessary ingredients for the infusion broth of Wagner et al. (1925) and Dack and Wood (1928). Type A toxin production in the medium of Lewis and Hill (1947) was sufficient to enable crystallization of the toxin for the first time by Lamanna et al. (1946). A similar medium was developed by Nigg et al. (1947) for preperation of a type A toxoid in which no allergenic substances were desired.

In this study, three batches of crystalline type A toxin were purified using a modification of the method of Duff et al. (1957). The three batches were characterized at each step in the purification procedure for toxicity, % solids, absorbance values at 260 and 278 nm, and toxin yield. The proteins present after each step were analyzed by SDS-PAGE to assess purity of the toxin complex. One batch of toxin was selected for further analysis with regard to RNA content of the toxin at various steps in the purification process. Toxin production and recovery was variable with different individual peptones in the medium indicating that nutrition affected toxin quality.

Materials and Methods

Bacterial strains and culture production.

The Hall A strain of type A Clostridium botulinum was used to produce type A toxin complex. This strain was selected because of the high toxin titers it produces as well as the high degree of cell lysis observed during culturing. This strain was originally obtained from Dr. J. H. Mueller at Harvard University and was further screened for high toxin titers by Dr. E. J. Schantz and coworkers at Fort Detrick, MD. Stock cultures of C. botulinum Hall A were grown statically in 15 ml Hungate tubes containing 10 ml of cooked meat medium + 0.3% dextrose (CMM; Difco Laboratories, Detroit, MI) under an anaerobic atmosphere (80% N_2, 10%CO_2, 10%H_2) at 37°C for 24 h and frozen at -20°C until use. CMM cultures of the Hall A strain routinely gave toxin titers in excess of 10^6 intraperitoneal LD_{50}/ml (LD_{50}/ml) in 48 h.

Cultures for toxin purification were grown statically in either 12 or 15 liter volumes of 2.0% trypticase peptone (BBL, Cockeysville, MD), 0.75% bacto-peptone (Difco Laboratories, Detroit, MI), 1.0% yeast extract (Difco), 0.5% glucose, pH 7.4, in glass carboys of 15 or 20 liters at 37°C until such time as the culture was harvested (usually 5-7 days). Alternatively, some cultures were grown in 1-2 liter of 2.0% casein hydrolysate (Sheffield Inc., Norwich, NY), 1.0% yeast extract (Difco), 0.5% glucose, pH 7.4, in Erlenmeyer flasks. Glucose was autoclaved separately in a 50% solution and aseptically added to the remainder of the medium after cooling.

Toxin purification.

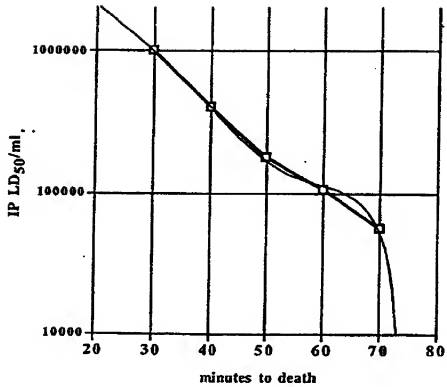
Type A toxin complex was purified in the manner approved by the U. S. Food and Drug Administration which is a modification of the method of Duff et al. (1957). The method consists of growing type A C. botulinum Hall A for 5-7 days in the simplified

casein hydrolysate medium at 37°C. At this time the pH of the crude culture was lowered to pH 3.4 with 3N H_2SO_4 and stirring. The toxin as well as many other proteins as well as cellular debris in the culture form large flocs and precipitate under these conditions and settle to the bottom. The precipitated material containing the toxin is termed the first acid mud. Toxin at this point is stable for years when stored at 4°C. The precipitated material was separated from the supernatant by decanting and the precipitate allowed to further settle overnight at room temperature. The precipitate was collected by centrifugation at (4,100 x g for 20 min at 5-10°C) and washed once with dH_2O. The first acid precipitate was then homogenized in dH_2O and the volume brought to 925ml. The toxin was extracted by the addition of $CaCl_2$ to a final concentration of 75mM (75ml of a 1.0M $CaCl_2$ solution) and raising the pH to 6.5-6.8 with 1N NaOH. The extraction was continuously stirred at room temperature for 2-4 h at which time the extracted toxin was separated from the cellular debris by centrifugation at 16,000 x g for 20 min at 5-10°C. The toxin was then immediately reprecipitated by lowering the pH to 3.7 with 1N HCl. This is termed the second acid precipitation and is generally allowed to settle overnight at 4°C. Precipitated material containing the toxin was collected from this second acid mud by centrifugation (16,000 x g for 20 min at 5-10°C). Toxin was reextracted with constant stirring in 150ml of 50mM sodium phosphate, pH 6.8, for 2-4 h at room temperature. The extracted toxin was separated from the insoluble material by centrifugation (16,000 x g for 20 min at 5-10°C) and the volume of the toxin extract made up to 150ml with 50mM sodium phosphate, pH 6.8. The toxin extract was then cooled to 0-2°C in a glycerol:ethanol:H_2O constant temperature bath. Alternatively, this cooling step may be done directly in a refrigerated centrifuge. When the toxin cooled to below 2-3°C, 65ml of 50% ethanol (precooled to -20°C) was slowly added with stirring over a period of 30-45 min taking care not to let the temperature rise above 2-3°C. The toxin was then cooled to -5°C and allowed to stand overnight under these conditions. Ethanol precipitated toxin was recovered by

centrifugation (16,000 x g for 20 min at -5°C) and the supernatant discarded. A small quantity (ca. 25 ml) of 50 mM sodium phosphate, pH 6.8, chilled to ca. 0°C was immediately added to dilute the residual ethanol and the pellet gently homogenized in a further volume of 50 ml of 50 mM sodium phosphate, pH 6.8. Toxin was gently dissolved in a final volume of 75 ml of 50mM sodium phosphate, pH 6.8, at room temperature for 1-2 h. The solution was clarified by centrifugation (12,000 x g for 20 min at 5-10°C) and the final volume made to 75 ml with 50 mM sodium phosphate, pH 6.8. The toxin was crystallized for the first time by the slow addition (15 min) of 20 ml of 4M $(NH_4)SO_4$ with continuous stirring. The toxin crystallized in 1-4 days at 4°C at which time the crystals were collected by centrifugation (12,000 x g for 20 min at 5-10°C). Crystalline toxin was dissolved in ca. 50 ml of 50 mM sodium phosphate, pH 6.8, clarified by centrifugation (12,000 x g for 20 min at 5-10°C), and the volume brought to 75 ml with 50mM sodium phosphate, pH 6.8. The toxin was crystallized a second time in the same fashion by addition of 20 ml of 4M $(NH_4)SO_4$ and incubating at 4°C. In some cases a third crystallization was performed by the same procedure.

Toxin assays.

Toxicity of various preparations were estimated using 18-22g female, ICR strain mice and the intravenous method of Boroff and Fleck (1966). The time-to-death method is converted to intraperitoneal LD_{50}/ml using the standard curve shown in Figure 1. Toxin concentrations are adjusted in order that the time-to-death values lie in the linear portion of the curve (30-70 min). In most assays three mice are injected and an average time-to-death value calculated. In some cases five mice are used and the average time-to-death calculated. Where appropriate, toxin titers were further determined using the standardized dilution to extinction method of Schantz and Kautter (1978).



$$y = -29.5x^3 + 5.33x10^3x^2 - 3.22x10^5x + 6.67x10^6$$
$$r^2 = 1.000$$

Figure 1. Clostridium botulinum type A toxin (3X crystallized) time-to-death standard curve.

RNA extraction.

Whole culture samples. Samples obtained from one 15 liter batch of C. botulinum Hall A were taken at various time points during the incubation of the culture and at steps in the toxin purification procedure. Samples taken during the incubation of the culture had dithiothreitol (Boehringer-Mannheim Corp., Indianapolis, IN) added to a final concentration of 1mM; EDTA (Sigma Chemical Co., St. Louis, MO) added to a final concentration of 1mM, and RNase inhibitor (Boehringer-Mannheim) added at a level of 50 units/ml. [All solutions, buffers, and glassware were made in diethyl pyrocarbonate (DEPC) treated dH_2O (0.1% DEPC (Sigma) in dH_2O, incubated $\geq$8 h, then autoclaved) unless otherwise noted]. These samples were then frozen at -20°C until extracted. Extraction of RNA was done by adding 100 µg/ml of lysozyme (Sigma) to the thawed and mixed samples and incubating for 15 min at 37°C. Sodium dodecylsulfate (SDS) (0.5% w/v) and Proteinase K (Pro K) (100 µg/ml) (Bethesda Research Laboratories, Gaithersburg, MD) were added and the mixture incubated at 50°C for 1 h. One-tenth volume of 3 M sodium acetate (Sigma) was added and the solution transferred to DEPC-treated centrifuge tubes. The solution was extracted once with an equal volume of 65°C TBE equilibrated phenol (TBE= 45mM Tris-HCl, 45mM sodium borate, 1mM sodium ethylene diamine tetraacetate, pH 8.0. Sambrook et al., 1989) by vortexing for 10 sec and cooling on ice. The aqueous phase was transferred to new centrifuge tubes after centrifuging at 12,000 x g for 20 min at 4°C and an equal volume of isopropanol added. Tubes were stored overnight at -20°C. Faint pellets visible after centrifugation at 12,000 x g for 60 min at 4°C were dissolved in 300 µl of dH_2O containing 50 units of RNase inhibitor and 35 units of DNase I (BRL). Following incubation at room temperature for 1.5 h three volumes of 4 M ammonium acetate, pH 4.5, were added and the mixture held on ice for 1 h. The pellets recovered by centrifugation at 12,000 x g for 30 min at room temperature were dissolved in 50 µl of dH_2O and reprecipitated by adding one-tenth

volume of sodium acetate, pH 4.5, 2.5 volumes 95% ethanol, and were stored overnight at -20°C. Following a wash with 70% ethanol, the pellets were dissolved in 100 µl dH_2O and extracted once with 1:1 phenol:$CHCl_3$ followed by a single $CHCl_3$ extraction. The RNA was precipitated with one-tenth volume of sodium acetate, pH 4.5, and 2.5 volumes 95% ethanol and incubated at -20°C for 2 h. The resulting pellets were dissolved in 50 µl of 1X TBE and quantitated by absorbance at 260nm assuming an absorbance of 1.0 = a 40 µg/ml solution of RNA.

RNA extraction from toxin purification samples.

Samples taken during the purification of crystalline type A toxin were treated in a similar fashion with the following exceptions; after Pro K digestion, 65°C phenol extraction, and isopropanol precipitation, the pellet was dissolved and the aqueous phase extracted with 1:1 phenol:$CHCl_3$ until no interface was visible (usually 2-4 times). The aqueous phase was then extracted once with $CHCl_3$. Samples from the first and second crystallizations were not treated with lysozyme.

SDS-gel electrophoresis.

Electrophoresis was performed using a Pharmacia Phast System (Pharmacia LKB Biotechnology, Piscataway, NY) and 12.5% linear pre-cast gels according to the manufacturers instructions. Sample buffer consisted of 75 mM Tris-HCl (Sigma), 5 M urea (Sigma), 5% SDS (Sigma), and 20% glycerol (Sigma), pH 6.8. All samples were boiled for 5-10 min. Some samples were reduced by the addition of 0.5% dithiothreitol. Bands were visualized by staining in 0.1% Coomassie brilliant blue R250 in 40% methanol, 20% acetic acid, destained in 25% methanol, 7.5% acetic acid followed by silver staining according to the procedure of Hammes (1990).

Agarose gel electrophoresis.

RNA samples were electrophoresed in 0.9% agarose-formaldehyde gels at 80V (constant voltage) according to the procedure of Sambrook et al. (1989).

Estimation of protein concentration.

Protein concentrations of crude extracts were estimated using the method of Smith et al. (1985) with bovine serum albumin as the standard. When working with purified forms of the toxin complex, protein concentration was estimated using the extinction coefficient at 278nm of 1.65 = 1 mg/ml of the toxin complex in a 1cm light path (Knox et al., 1970).

Results

Batch-to-batch characterization.

Purification of type A toxin complex using the U. S. Food and Drug Administration approved method of Duff et al. (1957) is shown schematically in Figure 2. Representative samples were taken at the various steps for analysis including optical densities, % solids, % recovery, and specific toxicity. These results are summarized in Table 1.

Production of crystalline type A toxin complex using the precipitation and crystallization method of Duff et al. (1957) appears to be a variable process (Table 1). Variation is introduced even before the seed culture is inoculated into the fermentation vessel; medium components play a very important role in the production and subsequent properties of the toxin. Using the same peptone from the same manufacturer but of a different lot had dramatic results on final yield of toxin (Table 2).

It is generally possible to recover 10-20% of the starting titer using the method of Duff et al. (1957) which compares favorably with the value of 17% originally reported by them. The final yields expected based on the starting titers and the assumption that the toxin in the crude culture had a specific activity of 30 LD_{50}/ng were: Batch 1, 13.7%; Batch 2, 11.4%; and Batch 3, 18.4%. Batch 1 had a yield of >25% of the starting toxicity after two crystallizations. However, the optical density (OD) ratio of 260/278nm was out of the accepted range of <0.6 recommended for high quality type A toxin complex for medical use (Schantz and Johnson, 1992). This necessitated a third crystallization in which another 12% of the toxin was lost. Recovery of toxin from one step to the next in the purification scheme was another source of variation. The acid precipitation steps at the outset were about 90% effective in recovering the toxin from solution. Toxin can be lost when extraction of the acid precipitates is incomplete, however, some losses are acceptable in the interest of time and simplicity. Repeated extractions of the acid mud can recover a

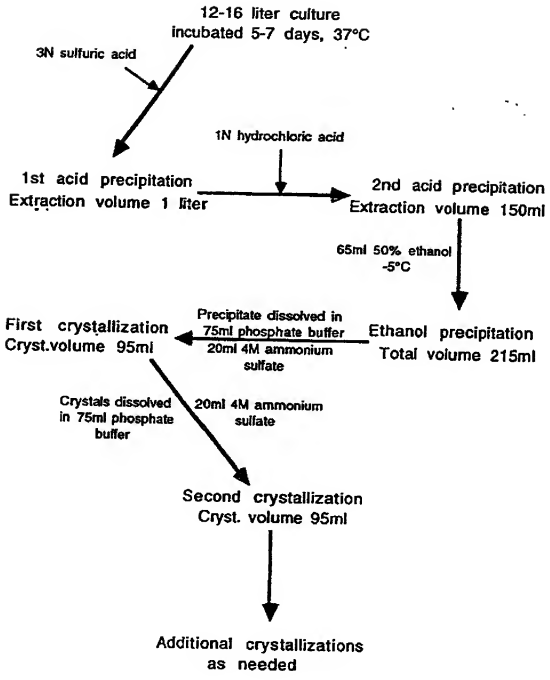


Figure 2. Clostridium botulinum type A crystalline toxin purification scheme.

Table 1. Batch-to-batch characterization of Clostridium botulinum Hall A purified according to a modification of the method of Duff et al., (1957).

	Batch #	5 day culture	1st acid ppt	2nd acid ppt	EtOH ppt supr	EtOH ppt pellet	1X cryst	2X cryst.	3X cryst
% solids	1	3.670	4.233	2.076	1.168	1.268	12.74	9.929	8.654
	2	2.731	2.664	0.879	0.435	0.557	10.76	6.831	
	3	2.745	1.612	0.906	ND	0.685	12.23	9.470	
260nm (1:20)	1	2.520	2.864	2.570	2.560	0.702	0.305	0.190	0.076
	2	2.113	1.499	1.024	1.470	0.159	0.149	0.035	
	3	2.109	2.612	1.699	ND	0.258	0.248	0.071	
278nm (1:20)	1	2.310	2.700	2.910	2.680	0.717	0.323	0.250	0.133
	2	1.683	1.109	0.932	0.913	0.186	0.171	0.060	
	3	1.727	2.146	1.071	ND	0.308	0.318	0.129	
ratio (260/278nm)	1	1.09	1.06	0.88	0.99	0.98	0.94	0.76	0.57
	2	1.26	1.35	1.10	1.61	0.86	0.87	0.58	
	3	1.22	1.22	1.59	ND	0.84	0.78	0.56	
BCA (µg/ml)	3	7600	5540	2160	1620	3810	1580		
total mg toxin	1	1100[a]					376	285	151
	2	600[a]					195	68.4	
	3	800[a]					362	147	
toxicity	1	2.2x10^{6}[b]					12[c]	22[c]	20[c]
	2	1.5x10^{6}[b]					11[c]	18[c]	
	3	2.0x10^{6}[b]					19[c]	28[c]	

[a]total mg are estimated using 30 LD_{50}/ng of toxin complex; [b]toxicity is given in LD_{50}/ml of crude culture; [c]toxicity is given in LD_{50}/ng of toxin complex.

Table 2. Toxin production by Clostridium botulinum Hall A after 7 days in various casein hydrolysates and recovery following acid precipitation.*

Peptone[1]	Toxin titer after 7 days[2]	pH after 7days	Titer after lowering pH to 3.4[3]	%ppt'd[4]
TT lot# 9NC29	1.36×10^6	5.76	6.0×10^4	95.6
TT lot# 1NB05	1.16×10^6	5.89	1.9×10^5	83.6
TT lot# 0NL30	8.8×10^5	5.85	1.8×10^5	79.5
B lot# 0ND05A	9.6×10^5	5.73	2.2×10^5	77.0
B lot# 2ND20	1.5×10^6	5.53	6.8×10^5	54.7
EKC lot# 1ND10A	1.6×10^6	5.53	$<4 \times 10^4$	>97
EKC lot# 0NK19	1.16×10^6	5.88	2.1×10^5	82.1
EKC lot# 1NK18	6.4×10^5	5.87	$<8 \times 10^3$	>95
A lot# 1NDO1V	9.6×10^5	5.78	1.1×10^5	88.5

*Medium consisted of 1 liter of 2.0% casein hydrolysate, 1.0% yeast extract (Difco), 0.5% glucose, pH 7.4.

[1]All peptones were obtained from Sheffield Laboratories, Inc., Norwich, NY.

[2]Toxicity was estimated using the intraveneous method of Boroff and Fleck (1966).

[3]Toxicity of supernatants was determined after acidification with 3N HCl to pH 3.4 and allowing precipitate to settle for 24 h.

[4]% precipitated = starting titer - supernatant titer/starting titer X 100.

higher percentage of the toxin (data not shown). Crystallization of the type A toxin complex results in losses due to the fact that not all of the toxin present associates into crystalline form (Sugiyama et al., 1977). A scanning electron micrograph of crystalline type A toxin complex is shown in Figure 3. Losses of toxin from one crystallization to the next are generally on the order of one-third to one-half of the total. In this study losses ranged from 32-65% of the total amount of toxin present from one crystallization to the next. The benefit gained from additional crystallizations is usually a decrease in the absorbance ratio (260/278nm) and generally an increase in specific toxicity. However, decreases in specific activity are not uncommon presumably due to increased handling of the toxin.

Specific activity of the final product is extremely important in pharmaceutical formulations. The higher the specific activity the fewer the number of nanograms required in a given vial since each vial (for sale in the U. S.) contains 100 LD_{50}. Decreasing the quantity of toxin injected is desirable since there are currently patients who are producing neutralizing antibodies to the toxin (Jankovic and Schwartz, 1991). From Table 1 it can be seen that final specific toxicities were variable. Average specific toxicities for these toxin batches as well as subsequent batches not described were in the range of 18-28 LD_{50}/ng. These toxicities are somewhat lower than those which can be routinely obtained using chromatographic purification methods, in which the specific activity of the toxin is usually in excess of 30 LD_{50}/ng of toxin complex (Sugiyama et al., 1977).

SDS-PAGE analysis of toxin samples.

Samples taken at the various stages of type A toxin purification shown in Figure 2 were examined using reducing and non-reducing sodium-dodecylsulfate gel electrophoresis. The results are shown in Figures 4-7. The final crystallizations of the three batches are compared side-by-side in Figure 8.

Figure 3. Scanning electron micrograph of crystalline type A toxin magnified 10,000X. The crystal was ca. 6 µm in length.

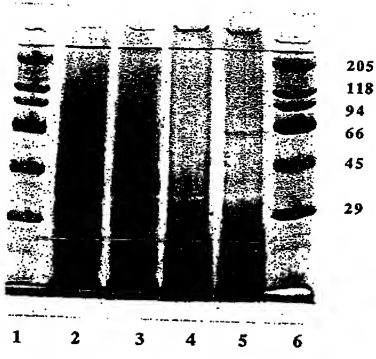


Figure 4. SDS-PAGE of whole culture and first acid precipitation samples taken during purification of C. botulinum type A toxin complex, batch 2. Lanes 1 and 6, molecular weight markers (rabbit myosin- 205 kDa, ß-galactosidase- 118kDa, E. coli phosphorylase b- 94 kDa, bovine serum albumin- 66 kDa, ovalbumin- 45 kDa, and bovine erythrocyte carbonic anhydrase- 29 kDa) (Sigma), 4-5 µg protein total. Lane 2, 5 day whole culture (unreduced); lane 3, 5 day whole culture (reduced with 0.5% w/v dithiothreitol); lane 4, extract of first acid precipitate (unreduced); lane 5, extract of first acid precipitate (reduced), 4-6 µg protein each lane.

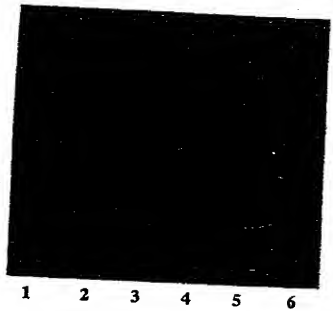


Figure 5. SDS-PAGE of extract from second acid precipitation and supernatant from ethanol precipitation taken during purification of C. botulinum type A toxin complex, batch 2. Lanes 1 and 6, molecular weight markers (rabbit myosin- 205 kDa, ß-galactosidase- 118kDa, E. coli phosphorylase b- 94 kDa, bovine serum albumin- 66 kDa, ovalbumin- 45 kDa, and bovine erythrocyte carbonic anhydrase- 29 kDa) (Sigma), 4-5 µg protein total. Lane 2, extract of second acid precipitate (unreduced); lane 3, extract of second acid precipitate (reduced with 0.5% w/v dithiothreitol); lane 4, supernatant from ethanol precipitation (unreduced); lane 5, supernatant from ethanol precipitation (reduced), 4-6 µg protein each lane.

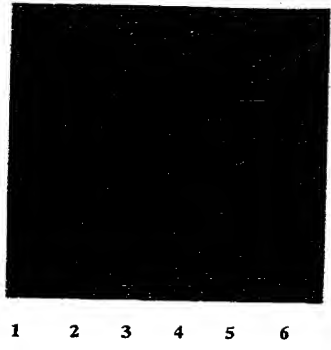


Figure 6. SDS-PAGE of pellet from ethanol precipitation and first crystallization taken during purification of C. botulinum type A toxin complex, batch 2. Lanes 1 and 6, molecular weight markers (rabbit myosin- 205 kDa, ß-galactosidase- 118kDa, E. coli phosphorylase b- 94 kDa, bovine serum albumin- 66 kDa, ovalbumin- 45 kDa, and bovine erythrocyte carbonic anhydrase- 29 kDa) (Sigma), 4-5 µg protein total. Lane 2, pellet from ethanol precipitation (unreduced); lane 3, extract from pellet of ethanol precipitation (reduced with 0.5% w/v dithiothreitol); lane 4, 1X crystallized toxin complex (unreduced); lane 5, 1X crystallized toxin complex (reduced), 4-6 µg protein each lane.

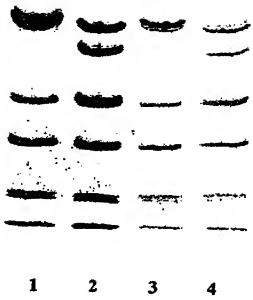


Figure 7. SDS-PAGE of 2X crystallized toxin complex taken during purification of C. botulinum type A toxin complex, batch 2 compared to type A toxin complex purified by the chromatographic method of Tse et al. (1982). Lane 1, 2X crystallized type A toxin complex (unreduced); lane 2, 2X crystallized type A toxin complex (reduced with 0.5% w/v dithiothreitol); lane 3, type A toxin complex purified by the method of Tse et al. (1982) (unreduced); lane 4, type A toxin complex purified by the method of Tse et al. (1982) (reduced), 4-6 µg protein each lane.

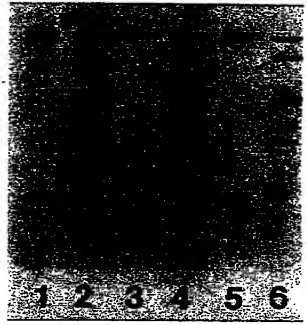


Figure 8. SDS-PAGE of toxin batches 1 (lanes 1, 2), 2 (lanes 3, 4), and 3 (lanes 5, 6). Samples in odd numbered lanes are unreduced, samples in even numbered lanes were reduced with 0.5% dithiothreitol.

Medium component dependence of toxin production.

The production of botulinum toxin was dependent on the medium used. The component of the growth medium which appeared to have the greatest bearing on final toxicity and recovery by acid precipitation was casein hydrolysate. Some of the differences observed in the batches of toxin shown (Table 1) were probably caused by the use of different lots of trypticase peptone. To further assess the role of casein hydrolysate on toxin recovery, 4 different types of casein hydrolysates comprosing 9 different lots were obtained. Each casein hydrolysate was used in a medium consisting of 2.0% casein hydrolysate, 1.0% yeast extract, and 0.5% glucose, pH 7.4, in a final volume of 1-2 liters. These were inoculated with 0.25 ml of the same 24 h old culture of C. botulinum Hall A. Incubation was carried out statically for 7 days at which time the cultures were assayed for toxicity and acidified with 3 N HCl (Table 2). The range of final toxicities between different lots of the same peptone varied from 8.8×10^5-1.36×10^6 LD_{50}/ml for NZ amine TT, 6.4×10^5-1.6×10^6 LD_{50}/ml for NZ amine EKC, and 9.6×10^5-1.5×10^6 LD_{50}/ml for NZ amine B. The only sample of NZ amine A used gave a final toxin titer of 9.6×10^5 LD_{50}/ml. High toxin titers were not attributable to final pH values following culture as hydrolysates which produced toxin titers in excess of 10^6 LD_{50}/ml had final pH values from 5.53-5.89 while those producing less than 10^6 LD50/ml had final pH values of 5.73-5.87.

In addition to high toxin titer, another important criterion for peptones intended for toxin production is recovery of the toxin during the first step of purification. When the pH is lowered to 3.4 to precipitate the toxin, the toxin should form large flocs and settle rapidly (<1 h after final acid addition). The casein hydrolysates varied in the degree to which the toxin precipitated after culturing (Table 2). For example, NZ amine B lot# 2ND20 gave the highest toxin titer among the hydrolysates tested but also gave the lowest recovery of toxin following acidification. The opposite was also observed: NZ amine EKC lot#

INK18 gave more than 95% recovery but initially produced only 40% as much toxin as produced in the NZ amine B lot# 2ND20. These results clearly show that casein hydrolysate markedly affected production and recovery of toxin and that more work is necessary to further elucidate the factors involved in toxin production.

RNA content of toxin.

RNA is well known to be associated with type A botulinum toxin complex (Tse et. al, 1982; Schantz and Johnson, 1992), and we were interested in determining whether specific classes of RNA were bound to the complex and carried through the protein purification. RNA was extracted from cultures of C. botulinum Hall A at different time points during incubation (Table 3). The extraction procedure recovered RNA which was analyzed on a 0.9% agarose-formaldehyde gel according to the procedure of Sambrook et al. (1989). The analysis showed 2-3 prominent bands presumably representative of ribosomal RNA (Figure 9, lanes 3-6). As the culture aged, the quantity of lower molecular weight species increased (Figure 9, lanes 5, 6). As the culture grew from early exponential phase to stationary phase (8 h-24 h), the amount of RNA also increased (Table 3). As the cells began to lyse at around 24-48 h, the amount of RNA dropped from a maximum of 106.6 µg RNA/ml of culture to a low of 10.6 µg/ml. RNA degrading enzymes are abundant in bacteria and the concentration of RNA in the culture medium should decrease following lysis.

One batch of toxin (batch 3) was assayed at various points in the purification procedure for RNA which might be associated with the toxin complex (Table 4). The amount of RNA associated with the toxin complex dropped during the purification. Approximately 3.4 µg of RNA/mg of toxin complex was detected in the 2X crystallized material or ca. 0.3% RNA/mg toxin. RNA was present as low molecular weight species

and none of the fragments were larger than ca. 800 bases with most less than 300 bases (Figure 10, lanes 1-7).

Table 3. Quantities of RNA extracted from Clostridium botulinum Hall A whole culture after various incubation times.

Sample	µg RNA/ml culture
8 h	49.6
24 h	106.6
48 h	25.5
120 h	10.6

Table 4. Quantities of RNA extracted from toxin preparations at various steps in purification of crystalline type A toxin, batch 3.

Sample	µg RNA/ ml sample	mg protein/ ml sample[1]	µg RNA/ mg protein	ratio (260/278)
2nd acid ppt	576	2.10	274	1.85
EtOH ppt/supr	292	1.62	180	1.85
EtOH ppt/pell	169	3.83	44	1.86
1X cryst/supr	44	3.88	11.3	1.90
1X cryst/pell	18.8	3.91	4.8	1.89
2X cryst	8	2.37	3.4[2]	1.88

[1]based on BCA assay data of total protein from batch 3, Table 1.

[2]batch 3 had a 260/278nm absorbance ratio of 0.56 and a specific toxicity of 28 LD_{50}/ng.

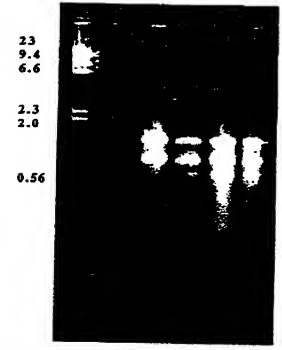


Figure 9. 0.9% agarose-formaldehyde gel of RNA from whole culture of Clostridium botulinum type A Hall strain. Lane 1, Hind III digestion of lambda DNA, (23 kb, 9.4 kb, 6.6 kb, 2.3 kb, 2.0 kb, 0.56 kb); lane 2, blank, lane 3, RNA extract from 8 h culture of Hall A, lane 4, RNA extract from 24 h culture of Hall A, lane 5, RNA extract from 48 h culture of Hall A, lane 6, RNA extract from 120 h culture of Hall A, ca. 1 µg total each lane.

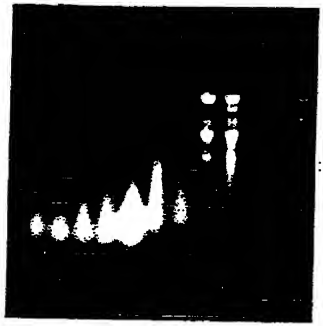


Figure 10. 0.9% agarose-formaldehyde gel of RNA from purification of toxin complex of Clostridium botulinum type A (Hall strain), batch 3. Lane 1, second acid precipitate; lane 2, ethanol precipitate (supernatant); lane 3, ethanol precipitate (pellet); lane 4, first crystallization (supernatant); lane 5, first crystallization (crystalline toxin); lane 6, second crystallization (supernatant); lane 7, second crystallization (crystalline toxin); lane 8, RNA extraction from 24 h culture Hall A; lane 9, RNA extraction from 120 h culture Hall A. Lanes 1-9 were each loaded with ca. 2-3 µg RNA extract each. Lane 10, blank; lane 11, BioRad low molecular weight RNA markers (1.6 kb, 1.0 kb, 0.6 kb, 0.4 kb, 0.3 kb), ca. 1 µg total; lane 12, BioRad high molecular weight RNA markers (7.4 kb, 5.3 kb, 2.8 kb, 1.9 kb, 1.6 kb), ca. 1 µg total.

Discussion

The purification of type A botulinum toxin for medical use has been done by a series of precipitations and crystallizations originally elucidated in 1957 by Duff and coworkers. The precipitation method was used to produce the only batch of toxin licensed so far in the United States by the Food and Drug Administration and was made by Dr. E. J. Schantz at the Food Research Institute in 1979 (Schantz and Johnson, 1992). This particular method was chosen in part because there would be no danger of other materials eluting from columns and contaminating the final product and possibly causing allergic reactions (Schantz and Johnson, 1992). Currently, it is possible to avoid such difficulties with careful attention to the column matrix, preparation of the column, and running conditions. Also, since stock suspensions of crystalline type A toxin can easily be kept at concentrations as high as10-20 mg/ml, diluting the toxin to therapeutic doses (ca. 10^6X) would tend to dilute most contaminants to non-reactive levels. There are more efficient methods of purifying protein available today including various types of column chromatography (Tse et al., 1982; Woody and DasGupta, 1988), and these methods should be considered for isolating toxin with high specific toxicity.

Different peptones (casein hydrolysates) were found in this study to affect production of type A toxin complex by the Hall A strain. The peptones were incorporated at 2.0% into a solution containing 1.0% yeast extract and 0.5% glucose, pH 7.4. There were significant differences between the various kinds of peptones as well as among lots of the same kind of peptone (Table 2). The mechanisms by which peptones affect toxin production and quality are not known. From the work done with C. tetani in the 1950's and 60's (Mueller and Miller, 1956; Latham et al., 1962), it is possible that a given peptone contains either a limiting peptide which limits the availability of an amino acid or that the peptone contains something which is inhibiting toxin formation. Recent work with

minimal media for C. botulinum has elucidated some of the requirements for toxin synthesis in group I (proteolytic) and group II (non-proteolytic) strains (Leyer and Johnson, 1990; Patterson-Curtis and Johnson, 1989; Whitmer and Johnson, 1988). For Group I C. botulinum, it was found that arginine and phenylalanine were essential for growth and toxin production. However, it was subsequently determined that high levels of arginine inhibited protease and toxin formation in both C. botulinum Hall A and Okra B (Patterson-Curtis and Johnson, 1989). High levels of tryptophan were shown to inhibit the production of type E botulinal toxin (Leyer and Johnson, 1990). Future work into the nutritional requirements of C. botulinum toxin formation may elucidate the reasons for the discrepancies observed between the various peptones.

Careful handling of C. botulinum during purification is critical for maximum recovery. The toxin is very susceptible to denaturation on the surface of air bubbles or sidewalls of the vessel due to agitation and in some instances temperature. There is usually some loss at each step in the purification due to these factors regardless of the purification method used. Maximizing the amount of toxin recovered is dependent mainly on the quantity produced by C. botulinum and the amount recovered in the first acid precipitation. The quantity of toxin produced is dependent on the lot of peptone used in the medium. The peptone composition also affects the flocculation and settling of toxin on acidification of the culture. One way around this problem of toxin staying suspended in the spent culture medium is centrifugation of the entire culture which is trivial if equipment such as continuous flow centrifuges are available but is very tedious and labor intensive if only standard centrifuges are present as cultures for bulk toxin production are typically 32-40 liters in size. Duff et al. (1957) reported that toxin also precipitated poorly when stock cultures of C. botulinum Hall A which had been stored at 4°C for an extended period of time and were subsequently used for toxin production. Cultures stored frozen at -20°C did not show this phenomenon suggesting that there was physiological modification of the

organism during refrigerated storage. The recovery of toxin on acidification appears to be related to the properties of the seed culture and also to the peptone composition.

When type A toxin complex is purified using the method of Duff et al. (1957), it is possible to maximize recovery at a few key steps. The rate of acid addition in both of the acid precipitations, the first to the crude culture, and the second to the extract of the first acid precipitate, does not seem to have any effect on either yield or the specific activity of the material recovered. Performing a second extraction of the first acid mud results in recovery of ca. 10-20% more toxin being recovered (data not shown). However, the 80-90% recovered in the first extraction is usually acceptable. The same results can be observed for the phosphate extraction of the second acid mud. The recovery at this step is lower (on the order of 60-70%). Subsequent reextraction of the pelleted material can recover an additional 10-15% but is usually not done.

Ethanol precipitation of the toxin is an extremely sensitive step in the purification. Toxin is denatured by ethanol at temperatures above ca. 2-3°C (Englard and Seifter, 1990). Great care must be taken to ensure that the temperature stays low during the exothermic addition of the 50% ethanol solution and that mixing is rapid. Recently, we found that doubling the volume of the ethanol precipitation from 215 ml of chilled toxin extract to 430 ml for large (>30 liter of crude culture) batches of toxin greatly increased the percent yield at this step. Two possible explanations for the ca. 5-8 fold increase in toxin recovered are that the ethanol as a precipitating agent was somehow limiting on the smaller scale or more likely that the larger volume of chilled toxin resists temperature fluctuation due to ethanol addition keeping the temperature low. Following the overnight -5°C incubation, the precipitated toxin is centrifuged from the ethanol solution and the ethanol decanted as rapidly as possible. Here, doing the ethanol precipitation step in the centrifuge itself means that the toxin does not have to be transferred from the precipitation vessel to a centrifuge bottle allowing the toxin to warm slightly. After the centrifugation step, the supernatant is

decanted and the pellet immediately covered with 0°C 50mM sodium phosphate buffer, pH 6.8, and homogenized with a glass rod. This step must be done quickly to ensure that residual ethanol present with the pellet is diluted to minimize denaturation of the toxin as the temperature rises. Toxin is unavoidably lost at this point due to surface denaturation from the mechanical stirring.

Crystallization of the toxin complex is accomplished by the addition of 4M ammonium sulfate to a final concentration of ca. 0.8-0.9M. This amount of ammonium sulfate is lower than that which causes which causes precipitation (ca. >50% saturated ammonium sulfate or 2.0 M). Presumably, the ammonium sulfate acts to reduce the ionic interactions between the toxin complex molecules to the point which favors protein-protein interactions necessary for alignment into a crystalline structure between individual toxin complex molecules. It may be that without ammonium sulfate present the ionic interactions are random causing arrangements unsuited to crystallization but with low concentrations of the salt present only interactions that are mediated by weaker, shorter range forces such as hydrophobic interaction and van der Waals forces are present which may allow proper alignment into a crystalline lattice.

The loss of toxin from one crystallization to the next is substantial (on the order of one-third to one-half). In order to minimize the number of crystallizations needed to lower the 260/278 nm absorbance ratio to the required 0.5-0.6 range and thereby reduce overall toxin loss, the crystallizations must be done slowly and allowed to go the completion. Addition of the 20 ml of 4 M ammonium sulfate to bring the solution to a final 0.84 M concentration should be dropwise with continuous stirring. The addition should take place over a period of ca. 30 min to ensure that the toxin is not precipitated due to ammonium sulfate concentration gradients forming. Incubation times of each crystallization are variable. The crystals usually begin to form overnight at 4°C and are visible as a clouding of the originally clear to slightly straw colored solution. The crystallization continues until

the crystals themselves precipitate usually within 7-14 days. Sugiyama et al. (1977) reported that complete crystallization was achieved in several months. After 7-14 days, the crystals (Figure 3) are separated from the mother liquor by centrifugation. The crystals are dissolved in 50mM sodium phosphate buffer, pH 6.8, and the absorbance ratio at 260/278nm determined. Toxin complex present in mother liquor which has been separated from the original crystals will continue to form crystals even after >14 days incubation in the first crystallization although the process is slow. This indicates that the crystallization process is protein concentration dependent with the majority of the crystals forming relatively quickly and falling out of solution. As the concentration of toxin complex in solution decreases, the rate at which crystals form also decreases.

Another explanation for the relatively poor recovery of toxin during crystallization using the modified method of Duff et al. (1957) was provided by Sugiyama et al. (1977). They propose that toxin complex which had been chromatographically purified prior to crystallization had a higher percentage recovery possibly due to lower interference from trace amounts of contaminating proteins. Recovery following crystallization of type A toxin complex was significantly higher (80% versus 42%) in their study when the toxic material was chromatographically purified instead of ethanol precipitated.

The two-fold goal in examination of RNA extracted from samples taken during purification was to determine the amount of RNA present in the purified toxin and to examine the RNA present to see if one species of RNA, perhaps an mRNA corresponding to that of the toxin gene was present in crystalline toxin. It can be imagined that such an mRNA could bind to the toxin complex and be carried through the purification procedure. RNA extracted from samples taken during toxin purification was compared to that obtained from 24 h and 120 h whole cultures of C. botulinum Hall A (Figure 9). Samples from whole cultures taken at 24 and 120 h showed large molecular weight bands of ca. 2.9 kilobases (kb), 1.5 kb, and 0.8 kb, along with smaller fragments of < 0.6 kb (Figure 9,

lanes 9, 10). These bands are the approximate molecular weights indicative of 23S and 16S rRNA. In contrast, samples taken during purification of toxin complex (Figure 9, lanes 2-8) showed mainly smears of <0.6 kb which are indicative of breakdown fragments of larger RNA molecules. The RNA purified from both the whole culture samples and the samples taken during toxin purification was probed in a Northern blot using a DNA probe to the toxin gene. The probe bound non-specifically to molecular weight markers and slightly to the 2.9 kb and 1.5 kb bands of the 24 h and 120 h whole culture samples but not to the samples taken during protein purification. This result indicates that if the RNA associated with the toxin complex is a specific messenger it is not representative of toxin mRNA or that it is present in very low amounts. The RNA that is associated with the complex is most likely rRNA that has been fragmented and associates non-specifically with the toxin complex in the culture.

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CHAPTER V

Stabilization and recovery of type A and B Clostridium botulinum neurotoxins following lyophilization

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Abstract

Botulinal toxin is used in very small quantities medically for the treatment of spastic muscle disorders due to its ability to inhibit motor-neuron signals to muscle fibers causing a flaccid paralysis. Botulinal toxin must be lyophilized or freeze-dried to allow for shipping and handling of the relatively delicate protein. Recovery of type A and B toxin activity following lyophilization was dependent on a number of factors. Conditions were found that gave >90% recovery of the toxicity following lyophilization of solutions containing 20-2,000 mouse 50% lethal doses. Recovery of toxicity following lyophilization of type A and B toxin complex as well as the purified ca. 150 kDa toxin molecules was obtained on drying 0.1 ml when the pH was maintained below 7.0 and serum albumins or other protein excipients were used as stabilizers in the absence of sodium chloride. Temperature stability of lyophilized type A toxin preparations was improved by addition of trehalose to the serum albumin system but not by addition of sucrose or maltotriose.

Introduction

Clostridium botulinum is a heterogeneous group of gram- positive, anaerobic, endospore forming, rod-shaped organisms that have in common the characteristic that they produce one of the most potent proteinaceous neurotoxins known. The endospores of C. botulinum are found worldwide in soils, marine, and freshwater environments. C. botulinum is classified phenotypically and by the serotype of toxin produced. There are seven serotypes of toxin currently recognized (A, B, C_1, D, E, F, and G) (Simpson, 1981). The individual toxins synthesized by a given serotype are found present along with non-toxic proteins some of which have hemagglutinating properties (Sugiyama, 1980; Sakaguchi, 1983; Schantz and Johnson, 1992). There is some antigenic cross-reactivity between the non-toxic proteins. This indicates that there is some amino acid sequence homologies forming common three dimensional epitopes among the non-toxic proteins of the complexes (Somers and DasGupta, 1991).

Neurotoxins produced by C. botulinum are large molecular weight proteins of ca. 150 kDa (Sugiyama, 1980). Type A botulinal toxin is initially produced as a single peptide chain or protoxin of 1295 amino acids in length (Binz, et al., 1990; Thompson et al., 1990). This protoxin must undergo post-translational proteolytic cleavage or nicking to achieve its characteristically high toxicity (Sugiyama et al., 1973). The nicking event occurs about one-third of the distance from the N-terminus. This nicking event generates the dichain molecule comprised of the 50 kDa light chain and 100 kDa heavy chain. The individual chains are connected by one disulfide bridge. The neurotoxin molecule exerts its characteristic muscle paralysis when the C-terminus region of the heavy chain binds to a receptor on motor-neuron end plates. The light chain is internalized through a channel formed by the N-terminus half of the the bound heavy chain, and causes an inhibition of neurotransmitter release by a mechanism involving proteolytic cleavage of vesicle

associated membrane proteins (VAMPs) (Schiavo et al., 1993). This lack of neurotransmitter signal across the synaptic junction to the muscle causes the flaccid paralysis seen in cases of botulism (Simpson, 1989).

Type A neurotoxin produced by C. botulinum is present as part of a complex of at least seven different noncovalently bound proteins (Somers and DasGupta, 1990). In culture media this toxin complex associates into dimers or trimers with a molecular weight of about 600 and 900 kDa, respectively (Tse et al., 1982; Habermann and Dreyer, 1986). The type A neurotoxin molecule has a molecular weight of 145 kDa (Gimenez and DasGupta, 1993) and in its fully active state consists of two separate peptide chains of 93 and 52 kDa that are connected by a disulfide link between cysteine residues 430 and 454 (Binz et al., 1990; Thompson et al., 1990). Type B toxin produced in culture is a mixture of two different size complexes. The larger or L complex has a molecular weight of ca. 500 kDa while the smaller or M complex is ca. 300-350 kDa (Kozaki et al., 1974; Sakaguchi, 1983). In contrast to the type A Hall strain, proteolytic type B strains do not fully activate the neurotoxic protoxin molecule associated with both complexes consequently a significant proportion of the toxin in the complex is present in the protoxin form. In these studies the two different size type B toxin complexes were not separated.

After some 23 years of development, a single batch of type A crystalline toxin complex was licensed by the Food and Drug Administration for medical use in the United States (Schantz and Johnson, 1992). This batch (#79-11) was produced at the University of Wisconsin-Madison, Food Research Institute in 1979 by Dr. E. J. Schantz, and is currently used in the treatment of hyperactive muscle disorders and dystonias due to its mode of action (Borodic, 1991; Jankovic, 1991; Scott, 1989). Disorders approved for treatment in the United States include blepharospasm, strabismus, and hemifacial spasm. Other dystonias being treated with type A toxin complex on an investigational basis worldwide include torticollis, aberrant regeneration of the seventh facial nerve, myofacial

pain syndromes, and others (Borodic, 1991). Treatment of patients involves injecting very small quantities (nanograms) of the toxin directly into affected muscle groups causing a regional decrease in muscle hyperactivity. High quality type A toxin complex for medical use has a specific toxicity of 3×10^7 mouse intraperitoneal 50% lethal doses per mg (LD_{50}) (Schantz and Johnson, 1992).

Botulinal toxin is very susceptible to denaturation due to surface denaturation, heat, and alkaline conditions. Lyophilization of botulinal toxin is the most economically sound and practical method of distributing the product in a form that is stable and readily used by the clinician. The current commercial type A botulinal toxin product is made by combining up to 500 ng/ml of type A toxin complex in 5.0 mg/ml human serum albumin (HSA) with 9.0 mg/ml sodium chloride at a pH of 7.3. After dissolution, 0.1 ml is dried to obtain 100 ± 30 active U of toxin, 0.5 mg of HSA, and 0.9 mg of sodium chloride per vial. This product has a saline concentration of 0.9 % when reconstituted in 1.0 ml of dH_2O. The current formulation gives considerable loss (up to 90 %) of activity during drying (Goodnough and Johnson, 1992) causing formation of inactive toxin that probably serves as a toxoid inciting antibody formation. In large dose applications, antibodies have been detected in patients that have become refractory to treatment (Greene, 1987; Borodic, 1991; Jankovic, 1991).

One goal of our research has been to improve recovery of active toxin following lyophilization. This reduces the amount of toxin required to obtain 100 active LD_{50} per vial. This improvement would also reduce the amount of inactive toxin in each vial and would lessen the possibility of antibody formation after injection of the preparation into patients. We have developed formulas which allow high recovery (>90%) of both type A and type B toxin complexes as well as purified type A and B neurotoxins.

The current commercial product must be stored at a temperature of -10°C or less to maintain the labelled potency for the one year shelf life. The product would be

considerably improved if conditions were developed that maintained shelf-stability at higher storage temperatures. This would facilitate more practical shipping and storage of the toxin. In this study, we have improved the freeze-drying formula of Goodnough and Johnson (1992) by adding carbohydrate excipients (chiefly trehalose) to increase the glass transition temperature of the dried material and thereby increasing the usable storage temperature. This improvement should enhance the temperature stability and lessen the risk of loss in potency with corresponding degradation and increase in antigenic potential.

Materials and Methods

Bacterial strains and culturing.

The Hall A strain of type A C. botulinum was used to produce crystalline type A complex. This strain was originally obtained from Dr. J. H. Mueller at Harvard University and was further screened for high toxin titers at Fort Detrick, MD by Dr. E. J. Schantz and coworkers. This strain is routinely used for production of type A botulinum toxin due to high toxin titers and the rapid onset of cell lysis (usually within 48 h). Type B toxin was produced from the proteolytic Okra B strain of C. botulinum. This strain was obtained from the Food Research Institute culture collection.

Stock cultures of C. botulinum Hall A and Okra B were grown statically in 15 ml Hungate tubes containing 10 ml of cooked meat medium + 0.3 % dextrose (CMM, [Difco Laboratories, Detroit, MI]) under an anaerobic atmosphere (80% N_2, 10%CO_2, 10%H_2) at 37°C for 24 h and frozen at -20°C until use. CMM cultures of the Hall A strain give toxin titers in excess of 10^6 LD_{50}/ml in 48-72 h. CMM cultures of the Okra B strain give toxin titers in the range of 5-9 x 10^5 LD_{50}/ml in 48-96 h. Type B toxin titers may be increased by trypsinization to increase the proportion of fully active nicked toxin molecules (DasGupta and Sugiyama, 1977). However, this procedure would necessitate the removal of trypsin and was not done in this study.

For toxin production, cultures of Hall A and Okra B were grown statically in 12-15 liter volumes of toxin production medium (TPM) consisting of 2.0% NZ TT (lot # 9NC29) casein hydrolysate (Sheffield Laboratories, Norwich, NY), 1.0% yeast extract (Difco), and 0.5% dextrose, pH 7.3-7.4, for 5-7 days at 37°C. Cultures of Hall A and Okra B showed heavy growth in this medium during the first 24-48 h followed by autolysis of the culture which was evident as a clearing and settling over the next 48-120 h. The Okra B strain did not produce toxin titers as high as Hall A nor did it lyse as rapidly or completely.

Type A toxin complex purification.

Type A toxin complex for use in drying studies was purified from culture broth using both the FDA-approved method involving precipitation and crystallization of the toxin complex (a modification of the method of Duff et al., 1957; see Chapter IV, this thesis) and by a method using preparative column chromatography (a modification of the method of Tse, et al., 1982). To prepare crystalline type A toxin, the 5-7 day culture was acidified with 3 N sulfuric acid to pH 3.4 and the resulting precipitate containing the toxin complex collected and extracted by adding $CaCl_2$ to a final concentration of 75 mM and raising the pH to 6.5-6.8. The extract was stirred for 2-4 h at room temperature and the toxin, now in solution, separated from the cellular debris by centrifugation (12,000 x g, 5-10°C, 20 min). The extracted toxin was reprecipitated by the addition of 1N HCl to a final pH of 3.7 and collected by centrifugation (12,000 x g, 5-10°C, 20 min). The toxin pellet was dissolved in 50 mM sodium phosphate buffer, pH 6.8, clarified by centrifugation (12,000 x g, 5-10°C, 20 min) and the volume made to 150 ml with buffer. Extracted toxin was cooled to 0-2°C in a constant temperature bath and 65 ml of 50 % ethanol precooled to -20°C added (15% final concentration) slowly with stirring over 30 min. The ethanol precipitate was incubated overnight at -2 to -5°C. The toxin was collected by centrifugation in a refrigerated centrifuge (12,000 x g, -5°C, 20 min) and the supernatant rapidly decanted. Residual ethanol was diluted by addition of 25-50 ml of 0°C 50 mM sodium phosphate buffer, pH 6.8, and the pellet gently homogenized with a glass rod. The toxin was dissolved to a final volume of 75 ml of the phosphate buffer, clarified by centrifugation (12,000 x g, 5-10°C, 20 min) and crystallized by the addition of 20 ml of 4 M ammonium sulfate (ca. 0.9 M final concentration). Toxin crystals were allowed to form at 4°C for 7-14 days. Crystals were collected by centrifugation (12,000 x g, 5-10°C, 20 min), redissolved in 75 ml of 50 mM sodium phosphate buffer, pH 6.8, and recrystallized by adding 20 ml of 4 M ammonium sulfate. The type A crystalline toxin used for these

studies was crystallized twice in the presence of 0.9 M ammonium sulfate and had a 260/278 nm absorbance ratio of 0.53. The extinction coefficient for type A toxin complex is A_{278} 1.65 = 1 mg/ml (Knox et al., 1970).

Type A toxin complex was also purified chromatographically by the method of Tse et al. (1982). Extracts of the first acid precipitated material were dialyzed against 50 mM sodium citrate, pH 5.5, and chromatographed at room temperature on a 1 liter DEAE-Sephadex A-50 column (Sigma Chemical Co., St. Louis, MO) equilibrated with the same buffer. One-tenth the column volume or less was chromatographed in a single passage with the toxin complex eluting in the first column volume without a gradient. Fractions from this protein peak which had a 260/278 absorbance ratio of less than 0.6 were pooled and precipitated by the addition of solid ammonium sulfate to ca. 60% saturation (39 g/100 ml).

Type A neurotoxin purification.

Type A neurotoxin was purified from the associated non-toxic proteins of the complex by a modification of the method of Tse et al. (1982). Toxin complex recovered from the DEAE-Sephadex A50, pH 5.5 column was precipitated by addition of 39 g of solid ammonium sulfate/100ml. The precipitated toxin complex was collected by centrifugation (12,000 x g, 5-10°C, 20 min), dialyzed against 25 mM sodium phosphate, pH 7.9, and applied to a DEAE-Sephadex A50 column equilibrated with the same buffer. The binding capacity of this particular matrix under these conditions is 0.9 mg of complex/ml of swollen gel. Various size columns were utilized by applying ca. 90% of the column binding capacity. Toxin was separated from the non-toxic proteins of the complex and eluted from the column with a linear 0-0.5M sodium chloride gradient. Toxin eluted from the column in the first peak and fractions which had 260/278 nm absorbance ratios <0.6 were pooled and precipitated by adding 39 g ammonium sulfate/100 ml. Material

recovered from the DEAE-Sephadex A50 column at pH 7.9 was further purified by chromatography on SP-Sephadex C50 at pH 7.0. Precipitated toxin from DEAE-Sephadex A50 columns at pH 7.9 was collected by centrifugation (12,000 x g, 5-10°C, 20 min) and dialyzed against 25 mM sodium phosphate, pH 7.0. The dialyzed toxin was applied to 25 ml SP-Sephadex C50 in 25 mM sodium phosphate, pH 7.0. Contaminating material did not bind to the column under these conditions. The toxin was eluted with a linear 0-0.25 M sodium chloride gradient.

Type B toxin complex purification.

Type B toxin complex was purified from crude culture fluid by a method involving the chromatographic procedure of Tse et al. (1982). Cultures of C. botulinum Okra B were acid precipitated to pH 3.4 using 3 N sulfuric acid. The acid mud was extracted once with 75 mM $CaCl_2$ and raising the pH to 6.5-6.8 analogous to purification of crystalline type A toxin. The clarified extracts were reprecipitated by lowering the pH to 3.7 with 1 N HCl. The second acid mud was then extracted with 50 mM sodium citrate buffer, pH 5.5, and the clarified extract dialyzed against the same buffer. A mixture of L and M type B toxin complexes were isolated by chromatography on 1 liter volumes of DEAE-Sephadex A50 equilibrated with 50 mM sodium citrate, pH 5.5. One-tenth the column volume or less was purified in a single passage with type B toxin complex eluting in the first column volume without a gradient. Fractions which had 260/278 nm absorbance ratios <0.6 were pooled and precipitated by addition of 39 g ammonium sulfate/100 ml. The extinction coefficient used for type B toxin complex was A_{278} 1.85 = 1 mg/ml (Beers and Reich, 1969). This pool represented type B toxin complex with a specific toxicity of 4.2×10^7 LD_{50}/mg.

Type B neurotoxin purification.

Type B neurotoxin was purified from the complex by a combined method involving the methods of Tse et al. (1982) and Moberg and Sugiyama (1978). Type B toxin complex in 25 mM sodium phosphate, pH 7.9, was applied to DEAE-Sephadex A50 (Sigma) equilibrated with the same buffer. Partially purified type B neurotoxin was eluted from this column with a 0-0.5 M sodium chloride gradient. Type B toxin fractions which had 260/278 nm absorbance ratios <0.6 were pooled and precipitated by the addition of 39 g ammonium sulfate/100 ml. Precipitated material was dialyzed against 25 mM sodium phosphate, pH 6.3, and applied to a pAPTG-Sepharose 4B column (p-aminophenyl-ß-D-thiogalactopyranoside) equilibrated with the same buffer (Sigma Chemical Co.). The charged column was washed with 5-10 column volumes of the loading buffer and the toxin eluted by changing the buffer system to 100 mM sodium phosphate, 1.0 M sodium chloride, pH 7.9. Fractions which had 260/278 nm absorbance ratios <0.6 were pooled and precipitated by addition of 39 g ammonium sulfate/100 ml.

Electrophoresis.

Protein samples were examined electrophoretically using the Pharmacia Phastsystem (Pharmacia LKB Inc., Piscataway, NJ) according to the manufacturers instructions. Precast 12.5% acrylamide gels (Pharmacia) were stained with 0.1% coomassie brilliant blue R250 in 16.7% acetic acid, 41.7% methanol. Gels were destained in 7.5% acetic acid, 25% methanol. Samples for electrophoresis were solubilized in 50 mM Tris-HCl, 5 M urea, 5% SDS, 20% glycerol, pH 6.8. Some samples were reduced by addition of dithiothreitol to a final concentration of 0.5%. All samples for SDS-PAGE were boiled for ≥5 min prior to electrophoresis.

Toxin assays.

Toxin titers were estimated in mice using the intravenous method of Boroff and Fleck (1966) and the intraperitoneal method of Schantz and Kautter (1978) in 18-22 g, female, ICR strain mice. Time-to-death values obtained from intravenous titration of type A and B toxin samples were converted to intraperitoneal LD_{50}/ml using a standard curve generated in our laboratory with type A complex. Botulinal toxin for titration was dissolved in 50 mM sodium phosphate, pH 6.8, and then further diluted as required in 30 mM sodium phosphate, 0.2 % gelatin, pH 6.4.

Lyophilization and excipients.

For lyophilization, toxin samples were diluted in the excipients to be tested (all excipients were from Sigma Chemical Co.), 0.1 ml or 0.5 ml aliquoted into 2 ml glass vials (Fisher Scientific Co., Pittsburgh, PA), the teflon lined screw cap closures fastened loosely, and the samples quickly frozen in liquid nitrogen. The frozen samples were placed into a lyophilization flask which was then immersed in liquid nitrogen. The flask was then connected to a laboratory freeze-drier (Virtis Freezmobile12, Virtis Co., Inc., Gardiner, NY). When the pressure dropped to ca. 30 mTorr, the liquid nitrogen jacket was removed. Pressure was maintained at or below 30 mTorr and condenser temperature was constant at -60ºC. Samples were allowed to come to room temperature and drying continued at ambient temperature over the next 18-24 h. At that time the flask was removed and the vials tightly capped. Vials were assayed for toxicity within 1-3 days (adapted from Goodnough and Johnson, 1992).

Some vials of lyophilized type A neurotoxin and type A toxin complex were stored at various temperatures to investigate the effect of added excipients on the shelf-stability of the dried material. In these cases, the tightly capped vials were placed into plastic bags,

sealed and stored at various temperatures (-20, 4, or 37°C) and the contents assayed for toxicity at various time points.

Results

Toxin purification.

Samples of crystalline type A toxin complex electrophoresed on sodium dodecylsulfate-polyacrylamide gels (SDS-PAGE) showed banding patterns typical for type A toxin complex (Figure 1, lanes 1, 2). Chromatographically purified type A toxin complex was electrophoretically equivalent to the crystalline material on SDS-PAGE (Figure 1, lanes 3, 4) and showed a higher specific toxicity than the crystalline toxin (3.2×10^7 LD_{50}/mg for the chromatographed material vs. 2.0×10^7 LD_{50}/mg for the crystalline toxin).

Type A neurotoxin purified from the non-toxic components of the complex showed a single band on unreduced SDS-PAGE gels of ca. 145 kDa indicating that it was free of contaminating non-toxic complex proteins (Figure 2, lane 1). Upon reduction of the disulfide bond with dithiothreitol, the two chains of the toxin migrated separately as the 93 kDa heavy chain and the 52 kDa light chain (Figure 2, lane 2). The purified neurotoxin had a specific toxicity of 9.0×10^7 LD_{50}/mg.

Unreduced SDS-PAGE samples of purified type B neurotoxin showed a single band at ca. 152 kDa indicative of type B neurotoxin (Figure 2, lane 3) (DasGupta and Sugiyama, 1976). Reduced samples showed three bands of ca. 152, 102, and 50 kDa (Figure 2, lane 4). The specific activity of the purified type B neurotoxin was 1.05×10^8 LD_{50}/mg.

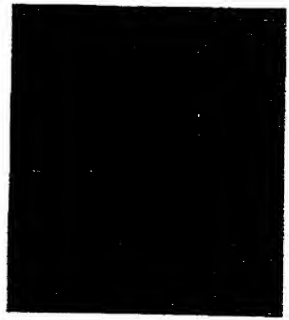

Figure 1. SDS-PAGE of Clostridium botulinum type A toxin complex purified by the modified method of Duff et al. (1957) (lanes 1 and 2) and by the method of Tse et al. (1982) (lanes 3 and 4). Lane 1- type A toxin complex (crystalline) (unreduced); lane 2- type A toxin complex (crystalline) (reduced with 0.5% w/v dithiothreitol; lane 3- type A toxin complex (chromatographically purified) (unreduced); lane 4- type A toxin complex (chromatographically purified) (reduced). 4-6 µg protein each lane.

Figure 2. SDS-PAGE of purified type A and B Clostridium botulinum neurotoxins. Lane 1- purified type A neurotoxin (unreduced); lane 2- purified type A neurotoxin (reduced with 0.5% w/v dithiothreitol); lane 3- purified type B neurotoxin (unreduced); lane 4- purified type B neurotoxin (reduced), ca. 4 μg each lane.

Stabilization of botulinal toxin during lyophilization.

Lyophilized preparations were usually reconstituted in 1.0 ml of distilled water. The use of 0.85% saline as a diluent gave equivalent results. The white cake dissolved immediately and was mixed by gentle inversion of the vials. The resulting solution was transparent. This solution was titrated by the same method used for the pre-lyophilization solution. Percent recovery values (calculated as number of mouse IP lethal doses/vial after lyophilization divided by number of mouse IP lethal doses/vial before lyophilization x 100) represent averages of trials done in at least duplicate. The variation in independent assays was ca. ± 20% as reported earlier by Schantz and Kautter (1978).

We initially determined whether recovery of active toxin following lyophilization was dependent on the rate of freezing and hence on ice crystal size. Freezing at -20°C or -70°C for 24 h in 50 mM sodium phosphate buffer, pH 6.8, resulted in slight loss of activity (75-90% recovery) compared to >90% recovery on flash freezing at -200° C in liquid nitrogen followed by 24 h storage at -70°C (data not shown). This could indicate that the larger ice crystals formed in the slower freezing cycle had a slighty negative impact on toxin stabilty by forming a larger more open structure when these crystals were sublimed during lyophilization. Previous studies in our laboratory also showed that no detectable inactivation (≤20%) of type A crystalline toxin (10^4 LD_{50}/ml) occurred during repeated freezing and thawing at -20°C in several buffers. These included 50 mM sodium phosphate (pH 6.2-6.8), 50 mM sodium succinate (pH 6.0), or 50 mM sodium citrate (pH 5.5) (Whitmer, Johnson and Schantz, unpublished). Freezing in 30 mM acetate buffer, pH 4.2, resulted in irreversible loss of toxicity (Schantz and Scott, 1981; Whitmer et al., 1987; Goodnough and Johnson, 1992).

The effect of salt concentration during freezing was examined for its effect on toxicity. Botulinal toxin is precipitated during various purification steps with ammonium sulfate at concentrations in excess of 60% saturation at room temperature with no loss of

activity. This is ca. equivalent to a 33% (w/v) solution of the salt. Sodium chloride at 0.9% in the commercial formulation may reach concentrations in excess of 6 M during lyophilization (Franks, 1990a) or ca. 35% (w/v) prior to crystallization. Freezing samples of type A toxin complex at -20ºC and -70ºC in 5.0 M solutions of sodium chloride, pH 6.2, did not affect toxin activity and full recovery was obtained after 24 h at both freezing temperatures and rates tested.

Lyophilization of type A toxin complex at 100-1,000 LD_{50}/vial (i.e. 3.3-33 ng/vial) in the absence of protein drying excipients gave almost complete loss of activity (Table 1, adapted from Goodnough and Johnson, 1992). It was not possible with such small quantities of toxin to determine if there were any losses due to aggregation since no visible precipitate formed when the dried material was reconstituted. The recovery of botulinal toxin activity following lyophilization was dependent on three factors. The formulation had to have a protein stabilizing agent present. In our case, serum albumins were most commonly used although other globular proteins such as gelatin, alpha-lactalbumin, and lysozyme worked in place of the albumins. The preparation for drying had to be free of sodium chloride and the pH of the preparation had to be maintained below 7.0 (Table 1). The results which stemmed from studies using crystalline type A toxin complex were then applied to other forms of botulinal toxin.

Type A toxin complex which was chromatographically purified gave identical recovery as the crystallized type A toxin complex following lyophilization. Chromatographically purified type B toxin complex (a mixture of the 300 kDa M and 500 kDa L complexes) was lyophilized under conditions which were favorable for recovery of type A toxin activity. Recovery of the chromatographically purified type B complex was the same as that attained with type A toxin complex (Table 2).

Table 1. Effect of excipients on recovery of toxicity of Clostridium botulinum type A toxin complex after lyophilization.

Excipients	Starting Toxin concentration[a]	pH	%recovery[b]
sodium phosphate[c]	50, 100, 1,000	5.0, 6.0, 6.8	<10
bovine serum albumin/ sodium chloride[d]	100	6.4	10
bovine serum albumin[e]	100, 1,000	6.4	88, 75
bovine serum albumin/citrate[f]	100, 1,000	5.0	>90, >90
bovine serum albumin/phosphate[g]	100, 1,000	5.5	>90, >90
bovine serum albumin/phosphate[g]	1,000	7.3	60
bovine serum albumin/phosphate[h]	1,000	6.0	>90
human serum albumin[i]	100, 1,000	6.4-6.8	>90, >90
alpha-lactalbumin[j]	1,800	6.1	>78
lysozyme[j]	1,800	5.3	>78
gelatin[j]	1,800	6.3	>78
bovine serum albumin/ trehalose[k]	500	5.7	>90
bovine serum albumin/ sucrose[l]	325	6.6	65
bovine serum albumin/ maltotriose[m]	250	7.0	>80

[a] Type A mouse IP lethal doses/vial before lyophilization; [b] %recovery = (number mouse lethal doses after lyophilization/number mouse lethal doses prior to lyophilization) x 100; [c] 50mM sodium phosphate; [d] bovine serum albumin (5.0mg/ml), sodium chloride (9.0mg/ml); [e] bovine serum albumin (9.0mg/ml); [f] bovine serum albumin (9.0mg/ml), 50mM sodium citrate; [g] bovine serum albumin (9.0mg/ml), 50mM sodium phosphate; [h] bovine serum albumin (9.0mg/ml), 50mM potassium phosphate; [i] human serum albumin (9.0mg/ml); [j] concentration = 9.0mg/ml; [k] 9.0mg/ml bovine serum albumin, 100mg/ml trehalose; [l] 9.0mg/ml bovine serum albumin, 250mg/ml sucrose; [m] 9.0mg/ml bovine serum albumin, 100mg/ml maltotriose. (adapted from Goodnough and Johnson, 1992).

Table 2. Recovery of activity following lyophilization of Clostridium botulinum type B toxin complex[a].

Excipient combination	Starting toxin concentration[b]	pH	%recovery[c]
bovine serum albumin/phosphate[d]	1,000; 100	6.0	>90
bovine serum albumin/phosphate[e]	1,000	6.0	>90
bovine serum albumin[f]	1,000	6.4, 6.8	>90
human serum albumin[g]	1,000	6.4	>90
gelatin[h]	1,000	6.2	>90

[a] type B complex was a mixture of 300 kDa and 500 kDa complexes (Sakaguchi, 1983); [b] mouse intraperitoneal lethal doses/vial; [c] (number of mouse lethal doses/vial after lyophilization + number of mouse lethal doses before lyophilization) x 100; [d] 9.0mg/ml bovine serum albumin, 50mM sodium phosphate; [e] 9.0mg/ml bovine serum albumin, 50mM potassium phosphate;

[f] 9.0mg/ml bovine serum albumin; [g] 9.0mg/ml human serum albumin; [h] concentration = 9.0mg/mL

In further efforts to reduce the amount of neurotoxin needed to yield 100 LD_{50}/vial following lyophilization, purified type A and B neurtoxins were dried in the presence of serum albumin. Although the neurotoxins are more labile than the complexes, recoveries on drying were similar to those obtained with the complexes (Table 3). The quantities of neurotoxin required to attain 100 LD_{50} of active toxin following lyophilization were ca. 1 ng per vial as compared to 3-8 ng per vial of the type A and B complexes. Recoveries of type A and B neurotoxin following lyophilization were high (≥80% of pre-lyophilized starting values) except in the samples which sucrose was added at a concentration of 250 mg/ml. The lower recovery rate (50%) with sucrose may have been related to a higher level of residual moisture as solutions containing high concentrations of sucrose are difficult to dry completely (Franks, 1990a). This residual moisture also had a deleterious affect on activity of selected type A botulinal toxin preparations when the different toxin preparations were stored at elevated temperatures (above -20°C) (Figures 3 and 4).

Table 3. Recovery of activity following lyophilization of purified Clostridium botulinum type A and B neurotoxins.

Excipient combination/ neurotoxin type[a]	Starting toxin concentration[b]	pH	%recovery[c]
bovine serum albumin/ type A	200	6.4	90
bovine serum albumin/ type B	100	6.4	80
human serum albumin/ type A	1,000	6.4	90
human serum albumin/ type B	100	6.4	90
bovine serum albumin, trehalose/ type A	500	5.7	>90
bovine serum albumin, sucrose/ type A	325	6.6	50
bovine serum albumin, maltotriose type A	250	7.0	90

[a] bovine and human serum albumin concentration was 9.0 mg/ml, carbohydrate concentration was 100 mg/ml in all cases except sucrose which was 250 mg/ml; [b] mouse intraperitoneal lethal doses/vial; [c] (number of mouse lethal doses/vial after lyophilization ÷ number of mouse lethal doses before lyophilization) x 100.

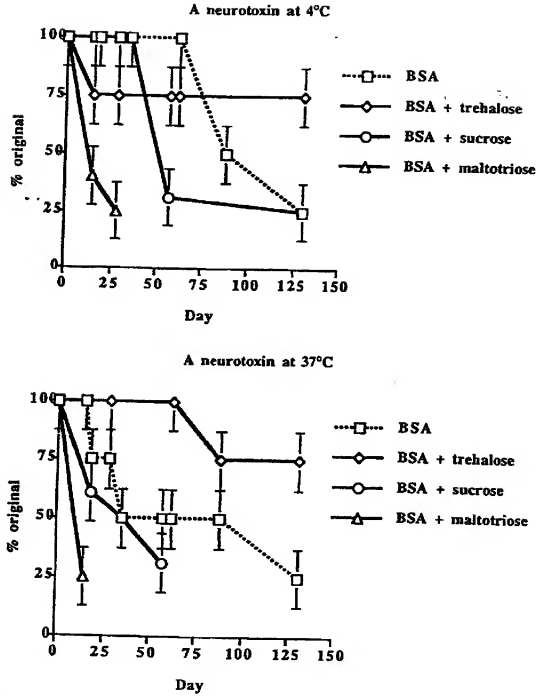

Figure 3. Stability of lyophilized purified type A neurotoxin at 4 and 37°C in the presence of bovine serum albumin and carbohydrate excipients.

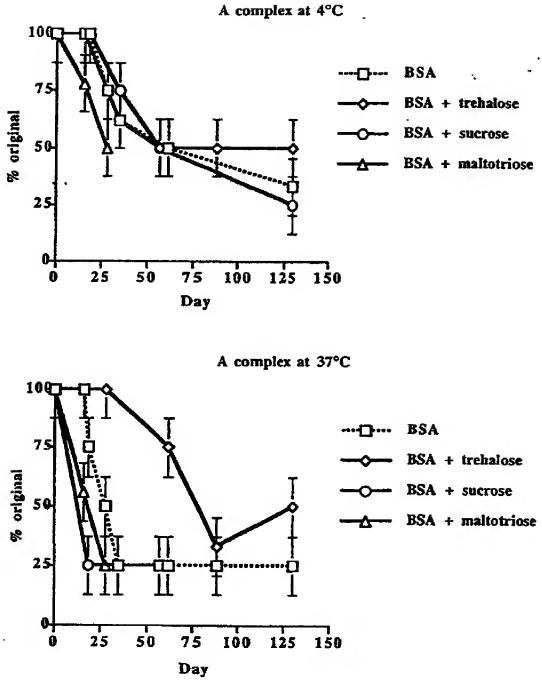

Figure 4. Stability of lyophilized type A toxin complex at 4 and 37°C in the presence of bovine serum albumin plus various carbohydrate excipients.

Stability of lyophilized botulinal toxin during extended storage.

Three different carbohydrate excipients were added to the bovine serum albumin system in an effort to raise the shelf-storage temperature by elevating the glass transition temperature of the dried material. Trehalose and maltotriose were added to the drying formulation at 100 mg/ml while sucrose was added at 250 mg/mL. Purified type A neurotoxin and crystalline type A toxin complex were used in separate experiments. The results with purified neurotoxin indicated that trehalose had a stabilizing effect at 4 and 37°C (75% activity retained after 130 days storage) which was much greater than the other two carbohydrates tested (≤25% activity retained for each) (Figure 3). Type A crystalline toxin complex was also stabilized by trehalose at the elevated shelf temperatures tested (50% retained activity after 130 days), but not to the same degree as that obtained with purified type A neurotoxin. Control vials for each combination of excipients were stored at -20°C and did not show any reduction in potency over the 130 days of incubation.

Discussion

The conditions used for lyophilization in this study had a considerable effect on recovery of active botulinal toxin. One of the most critical factors that contributed to recovery of active toxin was the omission of sodium chloride from the solution to be lyophilized. This omission combined with the presence of a protein excipient in large excess (1,000-10,000 fold excess) of the botulinal toxin concentration yielded >90 % of the starting toxicity when the pH was maintained below 7.0. Recovery of toxin activity following lyophilization was dependent to a more limited extent on the pH of the solution prior to lyophilization (Table 1). pH values of 7.0 and 7.3 were tested which did not give recovery rates as high as those obtained at pH values below neutral (Table 1). While freeze concentration and subsequent differential crystallization rates of the buffer components and salts present in solution, especially for sodium phosphate buffered systems, has been shown to alter the pH during lyophilization (Van den Berg, 1966; Pikal, 1990), the use of sodium phosphate or potassium phosphate in our experiments did not effect recovery of active type A toxin complex (Table 1). Solutions of BSA or HSA at 9.0 mg/ml had pH values of 6.4-6.8 at which full recovery was obtained. Full recovery of toxin activity was also obtained when the pH was adjusted to 5.0 or 5.5 by the use of sodium phosphate or sodium citrate buffered systems (Table 1). Recovery of activity following lyophilization of purified type A and B neurotoxin does not seem to be dependent on the presence of the non-toxic binding proteins of the complex as a high percentage of toxin activity was recovered using the same formulation as that used for the type A and B toxin complexes.

The differences in recovery rates dependent on pH could be due to the tendency for increased deamidation at higher pH levels. Deamidation with loss of activity has been demonstrated with other proteins such as lysozyme (Ahern and Klibanov, 1985), triose-phosphate isomerase (Ahern et al., 1987), calmodulin (Johnson et al., 1989a) and others

(Johnson et al., 1989b). Asparagine deamidates more readily than glutamine in model peptides with contributing factors being the primary amino acid sequence and the tertiary structure of the protein (Wright, 1991; Liu, 1992). Also, deamidation is more prevalent at asparagine-glycine and asparagine-serine sequences than at other asparagine sequences (Liu, 1992; Wright, 1991). There are at least six asparagine-glycine sequences in type A neurotoxin with three being in the light chain and three in the heavy chain. There are eight asparagine-glycine sequences present in the heavy chain region of the toxin (Binz et al., 1990). In other protein systems, these sequences were deamidated under certain drying and storage conditions (Wright, 1991). Further work will be done to determine whether deamidation is occurring in botulinal toxin during lyophilization and storage.

In most experiments in this study, 0.1 ml of botulinal toxin was lyophilized. This is equal to or less than the amount needed for most automated filling equipment in commercial lyophilization laboratories. When the fill volume was increased to 0.5 ml (with a subsequent reduction in serum albumin concentration to 1.8 mg/ml to maintain a post-lyophilization concentration of 0.9 mg/ml following reconstitution) slightly lower recoveries (60-80% of initial toxicity) were obtained on lyophilization (Goodnough and Johnson, 1992). This could be due to the fact that the same size 2 ml vials and lyophilization cycle were used causing the frozen cake to be thicker. This increased resistance to water vapor escape could have left more residual moisture in the freeze dried cake since the same lyophilization cycle was employed.

Shelf stability is an important property of protein pharmaceuticals. The present formulation of botulinal toxin must be stored below -10°C and ideally below -20°C following drying to retain potency of the dried toxin. One possible reason for the instability observed at ambient temperatures is that the glass transition temperature of human serum albumin and sodium chloride is around -10°C. Storage above this transition temperature would allow the residual moisture in the amorphous phase to interact with the

toxin molecule promoting degradative chemical reactions (Franks, 1990b). The glass transition temperatures of commercially dried pharmaceuticals containing protein can be elevated by the addition of certain carbohydrates. For instance, Franks (1990b) lists the glass transition temperatures of trehalose, sucrose, and maltotriose as 77°C, 57°C, and 76°C respectively. From these data one could assume that the choice of carbohydrate excipient would not be critical. However, the water contents on freeze-drying these carbonydrates are significantly different. Trehalose retains the lowest with 16.7% water followed by maltotriose (31.0%) and sucrose (35.9%) (Franks, 1990b). The higher percentage of water associated with the freeze-concentrated material would require a longer drying cycle to remove this water. The majority of the moisture in the initial formulation is removed relatively quickly during the primary drying portion of the lyophilization cycle. It is during this part of the cycle that the ice crystals formed during freezing are sublimed. Remaining moisture such as that associated with water of hydration and bound in crystalline structures must be removed in the much longer secondary drying portion of the lyophilization cycle.

One aspect of the shelf life of botulinal toxin complex which has not previously been investigated is whether the presence of the non-toxic binding proteins add stability to the toxin molecule in the lyophilized state. These non-toxic binding proteins add stability to the toxin molecule in solution and in the gastric tract. Buffered solutions of purified neurotoxin show breakdown fragments and a reduction in specific activity when incubated in solution at ambient temperature in only a few days. Solutions of toxin complex do not show these breakdown fragments and maintain specific activity for weeks (Goodnough and Johnson, unpublished data).

Our results indicate that purified type A neurotoxin is more stable at elevated temperatures than type A toxin complex when lyophilized in the three excipient systems tested. One possible explanation for this is that when toxin complex is lyophilized there is

more residual water present in close proximity to the toxin molecule itself due to the water of hydration from the non-toxic binding proteins of the complex. This water which is already in close proximity to the toxin molecule could be available for degradative reactions when the dried material is raised above its glass transition temperature. Another possible explanation is that the type A toxin complex preparations had a small amount of a protease(s) which copurified with the toxin complex while these contaminants were removed during purification of the neurotoxin. These degradative enzymes could inactivate the toxin when there was sufficient free water present. The pure form of the toxin was much more extensively chromatographed and could have had these low level contaminants removed. As a third possibility, one of the non-toxic binding proteins themselves could have some kind of enzymatic properties which may have contributed to the inactivation of the lyophilized complex.

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CHAPTER VI

Mechanisms of inactivation of Clostridium botulinum neurotoxin during lyophilization

Abstract

Treatment of hyperactive muscle disorders with the neurotoxin of Clostridium botulinum involves direct injection of afflicted muscle groups with the reconstituted toxin. Prior to distribution to clinicians, the toxin must be carefully quantitated and dispensed then freeze-dried or lyophilized to allow shipment and handling of this delicate protein. The various drying processes and formulations cause varying degrees of inactivation of the toxin and formation of toxoid. This toxoid adds to the antigenic burden of the material and further increases the chances of patients developing neutralizing antibodies. The biochemical processes which result in the formation of this toxoid can involve aggregation, deamidation, peptide bond hydrolysis, and oxidative degradation. In this study, we demonstrate in a model system using purified type A and B Clostridium botulinum neurotoxins that aggregation, deamidation, and peptide bond hydrolysis occur during lyophilization procedure causing decreases in the specific toxicity.

Introduction

Clostridium botulinum is a heterogeneous group of organisms that produce a very potent proteinaceous neurotoxin which binds to motor-neurons at presynaptic junctions and inhibits the release of acetylcholine causing a flaccid paralysis (Schantz and Johnson, 1992). The organisms comprising the group are characterized by differing degrees of proteolysis and by the serotype of neurotoxin produced. There are seven known serotypes A, B, C_1, D, E, F, and G. The neurotoxin is post-translationally processed to form the active dichain molecule. Post-translational "nicking" occurs ca. one-third the length of the protoxin molecule from the N-terminus generating two fragments connected by a disulfide bond. This dichain molecule is comprised of a light chain (ca. 50 kDa) which is the neurotoxic portion and a heavy chain (ca. 100 kDa) which is responsible for binding to the receptor on the motor-neuron and internalization of the light chain (Niemann, 1991). The exploitation of the flaccid paralysis caused by type A toxin has been used in the treatment of spastic muscle disorders in humans since about 1981 (Schantz and Johnson, 1992). The U.S. Food and Drug Administration licensed type A botulinum toxin for treatment of some of these disorders as an orphan drug in 1989.

Freeze-drying or lyophilization has become a standard method of stabilizing proteins which are susceptible to inactivation or possible microbial contamination when stored in dilute solutions. Freeze-drying subjects proteins to changes in temperature, increases in salt concentration, alterations in pH, and exposure to degradative reactions (Pikal, 1990; Franks, 1990; Liu, 1992). It has been shown that exposure of C. botulinum toxin to high salt concentrations or to low pH values during purification is not detrimental to toxin activity (Schantz, 1964; Schantz and Johnson, 1992). However, the neurotoxin is a fragile protein molecule and has been shown to fragment under relatively mild conditions (DasGupta and Tepp, 1991). Exposure of some pharmaceutical peptides to mildly alkaline

pH values and moderate temperatures leads to deamidation of the primary amino acid sequences of some pharmaceutical peptides (Johnson and Aswad, 1990; Johnson et al., 1989a; Johnson et al., 1989b; Manning et al., 1989). Asparagine and glutamine residues can react with succeeding glycine residues at physiologic pH values (pH 6-8) leading to the formation of a cyclic imide structure with the corresponding loss of an amine group from the side chain of the asparagine or glutamine. The cyclic imide can then break open to form a normal L-aspartic or glutamic acid linkage or the D-isomer of the aspartic or glutamic linkage. Isoelectric focusing and cation exchange has been used with model peptides or relatively small proteins to show differences in overall charge due to deamidation (Liu, 1992; Patel, 1993).

Other biochemical processes can lead to denaturation of protein pharmaceuticals during lyophilization. Aggregation of protein can occur during freeze-drying due to exposure of internal hydrophobic amino acid residues to the aqueous solvent and to hydrophobic surfaces such as at solvent-air interfaces (Sluzky et al., 1991). Under such conditions, the formation of intermolecular hydrophobic interactions is favored which causes aggregation of the protein.

Hydrolysis can also cause loss of activity of proteins during lyophilization. The peptide bond between aspartate and proline residues can hydrolyze at low pH values and ambient temperatures (Marcus, 1985). The hydrolysis of certain aspartate-proline sequences in the heavy chain of type A C. botulinum neurotoxin occurred at pH values as high as 5.0 (DasGupta and Tepp, 1991).

The presence of free water is necessary in all of these denaturation reactions. In lyophilized protein preparations, free water is generally present only at temperatures above that of the glass transition temperature of the material (Franks, 1990). The glass transition is the temperature at which the material changes from an elastic solid or glass, in which the water is held in place (i.e. only very slowly diffusable), to that of a more pliable or

deformable rubber material in which water is free to diffuse and participate in chemical reactions. The rate of these reactions is dependent on the final water content of the dried material as well as the glass transition temperature of the components in the formulation (Franks, 1990).

In this chapter, I have examined processes which cause inactivation of botulinal toxin during lyophilization. This work suggests that the drying formulation and process may be optimized to reduce inactivation and formation of toxoid.

Materials and Methods

Bacterial culture and toxin purification.

Clostridium botulinum Hall A strain was used for the production of type A toxin complex. This strain is routinely used for production of type A botulinum toxin due to high toxin titers and the rapid onset of cell lysis (usually within 48 h). C. botulinum Okra B strain from the Food Research Institute culture collection was used to produce type B toxin complex. Stock cultures of each organism were grown statically in 15 ml Hungate tubes containing 10 ml of cooked meat medium + 0.3 % dextrose (CMM, [Difco Laboratories, Detroit, MI]) under an anaerobic atmosphere (80% N_2, 10%CO_2, 10%H_2) at 37°C for 24 h and frozen at -20°C until use. CMM cultures of the Hall A strain gave toxin titers in excess of 10^6 intraperitoneal 50% lethal doses (LD_{50}/ml) in 18-22g white mice within 48-72 h while Okra B cultures gave toxin titers of 5-9 x 10^5 LD_{50}/ml of crude culture.

For toxin production, cultures of Hall A and Okra B were grown statically in 12-15 liter volumes of toxin production medium (TPM) consisting of 2.0% casein hydrolysate (Sheffield Laboratories, Norwich, NY), 1.0% yeast extract (Difco), and 0.5% dextrose, pH 7.3-7.4, for 5-7 days at 37°C. Cultures showed heavy growth in this medium during the first 24-48 h followed by autolysis of the cultures which was evident as a clearing and settling over the next 48-120 h.

Toxin purification.

Crystalline type A toxin complex was purified by a modification of the method of Duff et al. (1957) as described elsewhere (Schantz, 1964; Goodnough and Johnson, 1992; Schantz and Johnson, 1992). This method was identical to the method used to produce type A toxin complex for medical use in the United States. Purified type A neurotoxin was

purified by the method of Tse et al. (1982) with the addition of a final chromatographic step on SP-Sephadex C50 (Sigma Chemical Co., St. Louis, MO) at pH 7.0 according to the method of DasGupta and Sathyamoorthy (1984).

Type B neurotoxin was purified from crude culture by a method involving the chromatographic procedures of Tse et al. (1982) and Moberg and Sugiyama (1978). Briefly, cultures of C. botulinum Okra B were acid precipitated to collect the toxin from the whole culture. Type B toxin complex was separated from the remaining proteins in the extract of the acid precipitate on a 1 liter DEAE-Sephadex A50 (Sigma Chemical Co., St. Louis, MO) (5 cm x 65 cm) at a pH of 5.5 in 50 mM sodium citrate buffer. The neurotoxin was partially separated from the associated non-toxic proteins of the complex on a DEAE-Sephadex A50 column at pH 7.9 according to the method of Tse et al. (1982). The partially purified neurotoxin was purified to homogeneity by binding the non-toxic proteins of the complex to a pAPTG-Sepharose 4B column (p-aminophenyl-ß-D-thiogalactopyranoside) (Sigma) and eluting the purified toxin as a single peak according to the method of Moberg and Sugiyama (1978).

Electrophoresis.

Protein samples were examined by SDS-PAGE using the Pharmacia Phastsystem and precast, linear 12.5% polyacrylamide gels (Pharmacia LKB Inc., Piscataway, NJ) according to the manufacturers instructions. SDS-PAGE was also done with the BioRad Protean II system (BioRad Laboratories, Richmond, CA) using linear polyacrylamide gels according to the method of Laemmli (1970) as modified by Hames (1990). Gels were stained with 0.1% coomassie brilliant blue R250 in 16.7% acetic acid, 41.7% methanol and destained in 7.5% acetic acid, 25% methanol. Some gels were further silver stained according to the method Hames (1990). Protein samples for SDS-PAGE were solubilized in 50 mM Tris-HCl, 5 M urea, 5% SDS, and 20% glycerol, pH 6.8. Some samples were

reduced by addition of dithiothreitol to a final concentration of 0.5% (w/v). Samples for SDS-PAGE were boiled for 5-10 min prior to electrophoresis.

Proteolytic digestion and peptide mapping was done according to the method of Cleveland et al. (1977). For this analysis, protein bands were excised from the first SDS-gel after staining with Coomassie blue. The bands were cut out of the first gel using a razor blade and loaded directly onto the second gel.

Samples for native gel analysis were solubilized in 50 mM Tris-HCl, 20% glycerol, pH 6.8, and were run on pre-cast, 4-15% gradient gels according to manufacturers instructions (Pharmacia). Urea was added to some samples for native gel electrophoresis to a final concentration of 8 M. Samples for isoelectric focusing were dissolved and diluted in 25 mM sodium phosphate, pH 7.3, and run using pre-cast isoelectric focusing gels (Pharmacia) according to the manufacturers instructions.

Electrotransfer of SDS-PAGE gels for amino acid sequencing.

Proteins separated by SDS-PAGE using the BioRad Protean II system were transferred to Immobilon brand PVDF (polyvinylidene difluoride) membrane (Millipore Corp., Bedford, MA) according to the method of Matsudaira (1987). Transfers were made onto PVDF membranes in 10 mM CAPS (3-cyclohexylaminopropanesulfonic acid), 5% methanol, pH 10, at 30 mA constant current overnight (ca. 16 h) using the BioRad Transblot electrotransfer cell. The PVDF membranes were first briefly wetted with 100% methanol prior to assembly of the electrotransfer cassette. Following transfer, membranes were briefly (2-3 min) stained with 0.1% Coomassie brilliant blue R250 in 40% methanol and destained with 40% methanol to visualize transferred bands. Bands for amino acid sequencing were excised with a clean razor blade and sequenced at the University of Wisconsin Biotechnology Center, Madison, WI.

Toxin assays.

Toxin titers were estimated in mice using the intravenous method of Boroff and Fleck (1966) and the intraperitoneal method of Schantz and Kautter (1978) in 18-22 g, female, ICR strain mice. Time-to-death values obtained from intravenous titration of type A and B toxin samples in 3-5 mice were averaged and converted to intraperitoneal LD_{50}/ml using a standard curve generated in our laboratory for the type A complex. Botulinal toxin for titration was dissolved in 50 mM sodium phosphate, pH 6.8, and then further diluted as required in 30 mM sodium phosphate, 0.2 % gelatin, pH 6.4.

Deamidation assays.

Estimation of the isoaspartyl content of unlyophilized or lyophilized, and reconstituted purified A and B neurotoxins was done using a methanol diffusion assay of Macfarlane (1984) as modified by McFadden and Clarke (1986). Isoaspartyl-delta sleep inducing peptide (isoAsp-DSIP) (BACHEM Bioscience, Inc., Philadelphia, PA) was used as a standard. IsoAsp-DSIP has the sequence:

H-Trp-Ala-Gly-Gly-Asp*-Ala-Ser-Gly-Glu-OH

where Asp* is the residue with the isopeptide bond.

Isoaspartyl residues were methylated in a reaction volume of 50 µl at 30°C for 30 min in the presence of 2 µM protein methyltransferase (protein L-isoaspartyl methyltransferase type II; PIMT; E.C. 2.1.1.77 kindly provided by Dr. D. Aswad, University of California, Irvine) and 50 µM S-adenosyl-L-[*methyl*-^{3}H] methionine (500-600 dpm/pmol). The specific activity of the PIMT was 15-20 nmol/min/mg at 30°C using gamma-globulin as substrate or 25-30 nmol/min/mg using isoaspartyl-delta sleep inducing peptide isoAsp-DSIP. Methylation reactions were stopped by addition of 50 µl of 0.4 M

sodium borate, 5% SDS, 2.2% methanol, pH 10.0, and vortexing thoroughly. Twenty-five µl of individual reactions were spotted onto filter paper in the caps of 20 ml scintillation vials containing 10 ml of Ecolume (ICN, Costa Mesa, CA), the caps tightened, and the vials incubated at 40°C for 1 h. At that time the caps containing filter paper were replaced with new caps and the vials counted in a Beckman LS 5801 liquid scintillation counter.

Protein concentration determination.

Toxin concentrations were estimated using the extinction coefficients for type A toxin complex of A_{278} 1.65, purified type A neurotoxin of A_{278} 1.63 (Knox et al., 1970) and for both type B toxin complex and purified type B neurotoxin of A_{278} 1.85 = 1 mg/ml in a 1 cm light path (Beers and Reich, 1969). Protein concentrations were also estimated using the bicinchoninic acid method of Smith et al. (1985) with bovine serum albumin as the standard.

Amino acid sequencing.

Amino acid sequencing was done at the University of Wisconsin Biotechnology Center using an automated model 477A Liquid Pulse sequencer with on-line model 120A PTH analyzer and 610A data analysis system (Applied Biosystems, Foster City, CA).

Size exclusion high pressure liquid chromatography.

HPLC was performed with a Rainin HPXL system (Rainin Instrument Co., Woburn, MA). The size exclusion columns used were a Rainin SEC column (4.6mm x 250 mm) at a flow rate of 0.25 ml/min or a Dupont Zorbax GF-450 column (9.4 mm x 250 mm) at a flow rate of 1.0 ml/min. The isocratic solvent system in both cases was 100 mM sodium phosphate, pH 7.0, and protein was detected by absorbance at 278 nm. The

molecular weight cutoff for both columns was reported by the manufacturers to be 1-2 x 10^3 kDa.

Results

SDS-PAGE of botulinum toxin preparations.

The purity of type A toxin was evaluated by SDS-PAGE (Figure 1). Lane 2 contained 4-6 µg of purified type A neurotoxin with a specific toxicity of 96 LD_{50}/ng. A single major band is seen at 145 kDa. Lane 3 contained 4-6 µg of purified type A neurotoxin that was reduced with 0.5% (w/v) dithiothreitol. Two major bands are seen at 93 kDa (heavy chain) and 52 kDa (light chain). Lane 4 contained 4-6 µg of type A toxin complex and showed the characteristic 7 bands. Lane 5 contained 4-6 µg of type A toxin complex that was reduced with 0.5% (w/v) dithiothreitol.

Purified type B neurotoxin was analyzed by SDS-PAGE (Figure 2). Lanes 1 and 2 contained crystalline type A toxin complex (unreduced and reduced, 4-5 µg each lane) which was used as a molecular weight marker. Lane 3 contained 5-6 µg of purified type B neurotoxin, molecular weight ca. 152 kDa (unreduced). Lane 4 contained 5-6 µg of purified type B neurotoxin that was reduced with dithiothreitol (0.5% w/v) showing heavy (ca. 102 kDa) and light (ca. 50 kDa) chains. In contrast to type A, a substantial portion of type B neurotoxin remained in the unnicked form (152 kDa) after reduction of the disulfide bond connecting the individual chains.

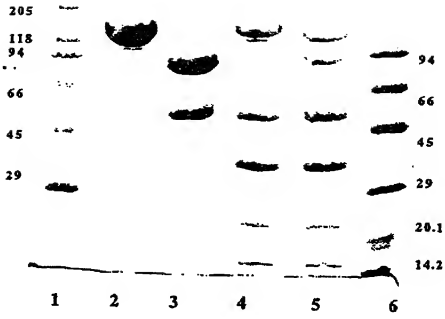


Figure 1. SDS-PAGE analysis of type A toxin preparations. Lane 1, molecular weight markers (in kDa); rabbbit myosin, 205, E. coli ß-galactosidase, 118, E. coli phosphorylase, 94 , bovine serum albumin, 66, ovalbumin, 45, carbonic anhydrase, 29, (Sigma Chemical Co., St. Louis, MO), 5-6 µg protein total; lane 2, purified type A neurotoxin (unreduced), 4-5 µg protein; lane 3, purified type A neurotoxin (reduced with 0.5% (w/v) dithiothreitol), 4-5 µg protein; lane 4, type A toxin complex (unreduced), 4-5 µg protein; lane 5, type A toxin complex (reduced), 4-5 µg protein; lane 6, molecular weight markers (in kDa); E. coli phosphorylase,94, bovine serum albumin, 66, ovalbumin, 45, carbonic anhydrase, 29, soybean trypsin inhibitor, 20.1 (doublet), and alpha lactalbumin- 14.2, (Pharmacia), 4-6 µg total.

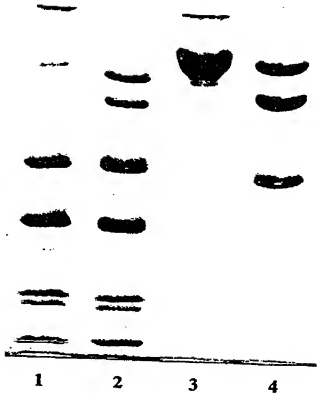


Figure 2. SDS-PAGE of type A toxin complex and purified type B neurotoxin. Lane 1, crystalline type A toxin complex (unreduced), 4-5 µg protein; lane 2, crystalline type A toxin complex (reduced with 0.5% (w/v) dithiothreitol), 4-5 µg protein; lane 3, purified type B neurotoxin (unreduced), 4-5 µg protein; lane 4, purified type B neurotoxin (reduced), 4-5 µg protein.

Aggregation of type A toxin during lyophilization.

Purified type A neurotoxin aggregated when lyophilized in the absence of protein (Table 1). In trial 1 Table 1, toxin was freeze-dried and stored at room temperature for several weeks before being reconstituted. In trials 2 and 3, aggregation was determined within 1-2 days following lyophilization. All trials represent the average of two separate determinations. The data showed that aggregation occurred as indicated by a decrease in soluble protein.

Native gel electrophoresis (Figure 3) of type A complex and purified type A neurotoxin supported the idea that aggregation occurred during lyophilization. Type A toxin complex (lane 1) migrated as a single polypeptide on 4-15% polyacrylamide native gels. When 8M urea was added to the toxin complex, some dissociation occurred (approximately 30%) (lane 2). Purified type A neurotoxin that had been lyophilized and treated with 8 M urea migrated as a single polypeptide. Purified type A neurotoxin that had been lyophilized but not treated with urea contained aggregates that did not enter the gel (lane 4).

Size exclusion chromatography of type A toxin complex by HPLC in 100 mM sodium phosphate, pH 7, at room temperature showed that unlyophilized or lyophilized toxin complex eluted with the same retention time. Following lyophilization, peaks indicative of breakdown products were not detected and peak broadening indicative of aggregation were not observed. However, since the toxin complex (900 kDa) was very nearly at the size exclusion limit of both commercially available columns (approximately 1-2 x 10^3 kDa in each case) it may not have been possible to detect large molecular weight aggregates using this system.

Table 1. Losses of purified type A neurotoxin on lyophilization.*

Sample	Trial	Specific activity[a]	Concentration (μg/ml) as determined by: A_{278}[b]	BCA[c]
pre-lyoph	1	88	100	131
	2	96	300	354
	3	94	200	220
post-lyoph[d]	1	60 (68%)	65 (65%)	80 (61%)
	2	80 (83%)	220 (73%)	237 (67%)
	3	75 (80%)	173 (87%)	175 (80%)

*In 50 mM sodium phosphate, pH 6.8, 0.10 ml fill volume. [a]LD_{50}/ng determined by i.v. method of Boroff and Fleck (1966); [b]absorbance at 278 nm using an extinction coefficient for type A toxin of $E^{1\%}$=16.3 in 1 cm light path; [c]BCA assay (bicinchoninic acid assay with an average of two determinations) using bovine serum albumin as standard (Smith et al., 1985) (Pierce Biochemicals, Rockford, IL, U.S.A.); [d]assayed after being dissolved in 1.0 ml dH_2O and centrifuged to remove aggregated protein. Numbers in brackets represent recovery percentages compared to pre-lyophilized values.

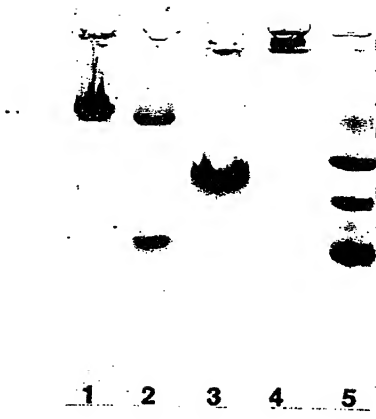


Figure 3. Native gel electrophoresis of unlyophilized type A complex and lyophilized, purified type A neurotoxin on 4-15% polyacrylamide gradient gel. Lane 1, unlyophilized type A toxin complex, 4-5 µg; lane 2, unlyophilized type A toxin complex plus 8M urea added to the sample buffer prior to electrophoresis, 4-5 µg; lane 3, lyophilized, purified type A toxin treated with 8 M urea prior to electrophoresis, 2-3 µg; lane 4, lyophilized, purified type A neurotoxin which was not exposed to urea, 4-5 µg; lane 5, markers consisting of thyroglobulin, ferritin, and catalase.

Deamidation of type A and B neurotoxins.

Deamidation was investigated as a source of denaturation using the vapor diffusion method of McFadden and Clarke (1986). Assays for deamidation showed that purified type B neurotoxin which had been lyophilized was a better substrate for the PIMT enzyme than its unlyophilized control (Table 2). Purified type A neurotoxin did not show this pattern and there was incorporation of radiolabel in both the unlyophilized and the lyophilized type A neurotoxin samples at approximately the same rate.

Isoelectric focusing was used in an attempt to determine differences in the charge of lyophilized toxin possibly due to deamidation during lyophilization. Purified type A toxin (Figure 4, lane 3) had a specific toxicity of ca. 90 LD_{50}/ng and a pI of 6.1. Upon lyophilization, there was no detectable shift in the isoelectric point of the toxin but the specific toxicity dropped to 65 LD_{50}/ng (Figure 4, lane 4).

Table 2. Incorporation of tritiated methyl groups by protein isoaspartyl methyltransferase into lyophilized and unlyophilized purified type A (Ant) and B (Bnt) neurotoxin.

Assay #	μM (toxin)	mol $^{*}CH_3$/mol toxin	
		Ant[a]	Ant (lyoph'd)[b]
	17.9	0.715	
1	20.3		0.613
	13.6	2.160	
2	13.0		2.345
3	13.3	2.088	
		Bnt[c]	Bnt (lyoph'd)[d]
	33.9	0.110	
4	40.5		0.290
	14.5	1.230	
5	8.5		3.689

[a]purified type A neurotoxin (unlyophilized), [b]purified type A neurotoxin (lyophilized, reconstituted with dH_2O, specific activity = 65 LD_{50}/ng), [c]purified type B neurotoxin (unlyophilized), [d]purified type B neurotoxin (lyophilized, reconstituted with dH_2O, specific activity = 53 LD_{50}/ng).

Figure 4. Isoelectric focusing gel (pH 3-9) of lyophilized and unlyophilized purified type A neurotoxin. Lanes 1, 5; broad pI markers (from the top of the gel down) consisting of trypsinogen, 9.30, lentil lectin-basic band, 8.65, lentil lectin-middle band, 8.45, lentil lectin-acidic band, 8.15, horse myoglobin-basic band, 7.35, horse myoglobin-acidic band, 6.85, human carbonic anhydrase B, 6.55, bovine carbonic anhydrase B, 5.85, ß-lactogloculin A, 5.20, soybean trypsin inhibitor, 4.55, and amyloglucosidase, 3.50 (Pharmacia), 8-10 µg protein; lane 2, sample buffer (25 mM potasium phosphate, pH 6.9); lane 3, purified type A neurotoxin, 4-5 µg protein; lane 4, lyophilized, purified type A neurotoxin, 4-5 µg protein.

Peptide bond hydrolysis.

Examination of type A toxin complex by SDS-PAGE before and after lyophilization indicated that no peptide bonds were broken (Figure 5). Lanes 1 (unreduced) and 2 (reduced with 0.5% w/v dithiothreitol) show the banding pattern of unlyophilized toxin complex. The pattern is typical of type A toxin complex showing toxin (lane 1, unreduced, 145 Kda, lane 2, reduced, 93 and 52 kDa) and the associated non-toxic binding proteins (118, 50, 35, 21.8, 20.8, and 17.5 kDa). Lanes 4 (unreduced) and 5 (reduced) show the pattern of polypeptides in the complex following lyophilization. The formulation used for drying contained 50 mM sodium phosphate, pH 7.3, 9.0 mg/ml sodium chloride, and 250 µg/ml of toxin complex with a specific activity of 28 LD_{50}/ng; 0.1 ml was lyophilized in 2 ml glass vials (Goodnough and Johnson, 1992). The lyophilized material had a specific activity of 7 LD_{50}/ng corresponding to a loss of about 70% of the starting activity. Analysis of the lyophilized preparation by SDS-PAGE did not show that peptide bonds in the toxin molecule were hydrolyzed with formation of breakdown fragments.

The possibility of peptide bond hydrolysis was further examined using purified neurotoxin. The simplified system of purified type A neurotoxin showed that peptide bond hydrolysis was occurring during lyophilization (Figure 6). The extent to which the hydrolysis occurred in the soluble material and the insoluble material in the reconstituted lyophilized cake was markedly different. Lyophilized type A neurotoxin was analyzed on a linear 12.5% polyacrylamide Phastgel (Pharmacia). The soluble material containing 3-4 µg of toxin soluble in dH_2O showed a doublet in the region of the light chain. The doublet occurred to a much larger extent in the insoluble, aggregated material (Figure 6, lane 5). The difference in molecular weights of the two bands is about 2 kDa. When the aggregated material following lyophilization was run on a longer gel (ca. 12 cm, 10% polyacrylamide gel using the BioRad Protean II system), the reduced toxin showed the 50-52 kDa doublet and the unreduced toxin showed a doublet at about 143-145 kDa (Figure 7).

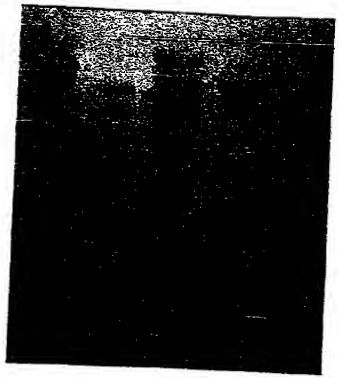


Figure 5. SDS-PAGE analysis of type A toxin complex before and after lyophilization. Lanes 1(unreduced) and 2 (reduced with 0.5% w/v dithiothreitiol), unlyophilized type A toxin complex, 4-5 µg protein; lane 3, molecular weight markers (in kDa); rabbit myosin, 205, E. coli ß-galactosidase, 118, rabbit phosphorylase b, 94, bovine serum albumin, 66, chicken egg albumin, 45, bovine erythrocyte carbonic anhydrase, 29, (Sigma), 5 µg protein; lanes 4 (unreduced) and 5 (reduced), lyophilized type A toxin complex, 4-5 µg protein.

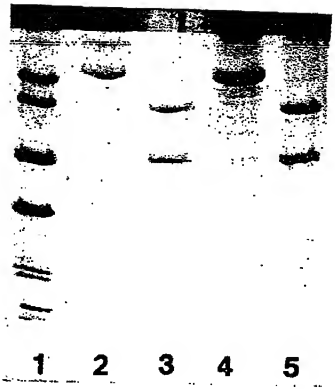


Figure 6. SDS-PAGE showing hydrolysis of purified type A neurotoxin during lyophilization. Lane 1, type A toxin complex (reduced with 0.5% dithiothreitol (w/v)), 4-5 µg protein; lane 2, lyophilized, purified type A neurotoxin, water soluble fraction (145 kDa), 3-4 µg protein; lane 3, lyophilized, purified type A neurotoxin, water soluble fraction (reduced) (93 kDa, 52 kDa), 3-4 µg protein; lane 4, lyophilized, purified type A neurotoxin, water insoluble fraction (145 kDa), 4-5 µg protein; lane 5- lyophilized, purified type A neurotoxin, water insoluble fraction (reduced), 4-5 µg protein.

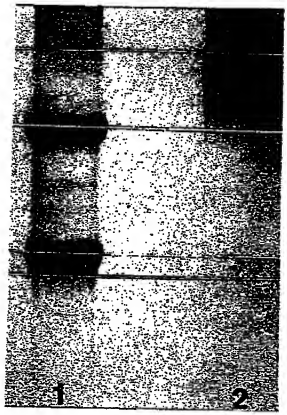


Figure 7. SDS-PAGE showing hydrolysis of light chain of type A neurotoxin during lyophilization. Lane 1, lyophilized, purified type A neurotoxin (reduced with 0.5% dithiothreitol (w/v)), 93 kDa, 52 kDa, 50 kDa, 7-10 µg total; lane 2, lyophilized, purified type A neurotoxin, 145 kDa, 143 kDa, 4-5 µg total.

The 50 and 52 kDa bands of the light chain doublet were separated on a 12 cm 12.5% SDS-PAGE gel and the individual bands excised and digested according to the method of Cleveland et al. (1977). The excised bands were digested with Streptomyces griseus pronase A and the fragments separated on a linear 15% polyacrylamide gel (Figure 8). The sample in lane 1 was digested with 16 ng of pronase, while lane 2 was undigested; both lanes showed the 52 kDa band. Lanes 3 (undigested) and 4 (digested with pronase) contained the second (50 kDa) band of the light chain doublet. The pattern of digestion fragments in lanes 1 and 4 are similar indicating homology between the 50 and 52 kDa bands. Lane 5 and 6 contained undigested 50 and 52 kDa bands while lane 7 contained 93 kDa heavy chain digested with pronase. Lane 8 contained 93 kDa heavy chain that was digested with 30 ng of trypsin while lane 9 contained the same 93 kDa heavy chain band digested with 15 ng of trypsin. The lack of homology between the digestion fragments in lane 7 and those in lanes 1 and 4 indicates that the origin of the 50-52 kDa doublet is from the light chain of the toxin molecule.

Figure 8. Partial proteolytic digestion of 52 kDa and 50 kDa light chain bands on 15% SDS-PAGE. Lane 1, 52 kDa type A neurotoxin light chain band excised from SDS-PAGE gel shown in Figure 7 plus 16 ng Streptomyces griseus pronase A; lane 2, undigested 52 kDa light chain band; lane 3, undigested 50 kDa light chain band; lane 4, 50 kDa type A neurotoxin light chain band excised from SDS-PAGE gel shown in Figure 7 plus 16 ng Streptomyces griseus pronase A; lane 5, undigested 50 kDa light chain band; lane 6, undigested 52 kDa light chain band; lane 7, 93 kDa heavy chain band from SDS-PAGE gel shown in Figure 7 plus 16 ng S. griseus pronase A; lane 8, 93 kDa heavy chain band from SDS-PAGE gel shown in Figure 7 plus 30 ng trypsin; lane 9, 93 kDa heavy chain band from SDS-PAGE gel shown in Figure 7 plus 15 ng trypsin.

Amino acid sequencing.

Amino acid sequencing was done on the 50 and 52 kDa bands to determine the residue at which hydrolysis was occurring. The bands were transferred to PVDF memebrane by the procedure of Matsudaira (1987). The 52 kDa band gave a sequence of P-F-V-N-K-Q-F-N-Y-K-x-P-V-N-G-V-D- upon Edman degradation. During the sequencing run, the signal strength dropped to near the resolution limit of the instrument after the valine 13 residue. The sequence was still readable after this but at a lower limit of detection. The 50 kDa band gave strong P and V signals in the first two cycles of Edman degradation but the remainder of the sequence was N-terminal blocked.

Discussion

Studying the mechanisms of inactivation of therapeutic levels of C. botulinum toxin during lyophilization and storage is difficult due to the fact that nanogram quantities of toxin are in each vial. In order to study possible denaturing mechanisms such as aggregation, peptide bond hydrolysis, and deamidation much larger quantities of the toxin had to be examined. It was found in this study that a self-protection effect occurred in which the toxin acted as its own stabilizing excipient when microgram quantities of toxin were lyophilized. Lyophilization of therapeutic levels of type A toxin complex (20-1,000 LD_{50}) using certain formulations resulted in a much larger percentage of toxin inactivated (>90% inactivated) (Goodnough and Johnson, 1992) than was found in this study (20-32% inactivated) using microgram quantities. Therefore, the exact mechanisms by which therapeutic levels of toxin are inactivated on drying are extrapolated from the data obtained with much larger quantities of toxin. This has been shown to be different from drying lower levels of toxin.

Aggregation of proteins occurs when the conformation of the molecule changes to allow more hydrophobic regions buried in the center of molecule to become exposed to the aqueous solvent system or other hydrophobic areas-thus favoring hydrophobic-hydrophobic interactions between individual protein molecules. Our results indicated that aggregation of toxin occurred during lyophilization. Toxin stored above its glass transition temperature (trial 1) could have allowed degradative chemical reactions such as deamidation and oxidation to occur. Storage in this condition may have allowed the samples to pick up water from the environment which was shown to cause further aggregation in other protein systems (Liu et al., 1991). The difference in recovery of material in trial 1, 2, and 3 may have been related to the smaller amount of material used in trial 1 giving a larger percentage of material which may have adhered to the vial. The difference in the specific activities

indicated that there could be additional inactivating events occurring during storage of the dried material. It is also possible that "self-protection" occurred in the trial with higher concentrations of toxin.

It is possible that there are oxygen dependent inactivation events occurring during lyophilization and storage of the dried toxin. The oxygen concentration in a partially frozen aqueous system at -3°C is 1,150 times higher than that at 0°C (Schwimmer, 1981). Cysteine residues have been shown to undergo auto-oxidation to form intra- or inter-molecular disulfide bonds as well as form oxidative degradation products such as sulfenic acid (Torchinsky, 1981). These oxidation reactions are greatly accelerated by the presence of metal ions such as copper and iron. No intra-molecular disulfide bond redistribution was seen in these studies as judged by non-reducing SDS-PAGE. However, it is possible that intermolecular disulfide bonds were rearranged causing inactivation of the toxin since such arrangements should not change the SDS-PAGE electrophoretic pattern.

Aggregation could be promoted by the dried material slowly picking up moisture from the environment. It has been shown with bovine serum albumin that moisture adsorbed by the solid, freeze-dried product caused aggregation in part due to thiol-disulfide interchange (Liu, et al., 1991). Other water dependent reactions such as deamidation and peptide bond hydrolysis would also be expected to be more prevalent in such cases.

Aggregation of the toxin complex is difficult to detect using standard size-exclusion chromatography as the native complex eluted near the exclusion limit. However, since there was no discernible broadening of the peak of lyophilized toxin it is possible that the conditions used (i.e. 100 mM sodium phosphate, pH 7.0) allowed dissociation of aggregates to the native MW of 800-900 kDa.

Deamidation appeared to be occurring in all cases. Unlyophilized type A and B neurotoxins as well as the lyophilized neurotoxins were substrates of the enzyme (Table 2). Type B neurotoxin seemed to be a better substrate than type A for the enzyme on a weight

basis. The assays were not done at low enough neurotoxin concentrations to quantitatively determine the number of isoaspartyl residues per toxin molecule. Low substrate concentrations are a requirement of the PIMT enzyme due to its extremely slow turnover rate (Johnson and Aswad, 1991). Further evidence for the existence of isoaspartyl residues came from amino acid sequencing of the hydrolyzed light chain. During sequencing of the 52 kDa light chain band, the signal was identical to the light chain of that of Binz et al. (1990) except for the absence of the first methionine residue. However, after the automated sequencer read through the first thirteen residues up to V13, the signal strength dropped substantially to a point just above background levels. The weaker sequence from valine-13 on to the wash out of the signal was identical to that published for type A light chain. Since pmol quantities of material were sequenced, it was not possible to quantitate the decrease in signal. The decrease in signal strength at valine-13 could be explained by a portion of the material being sequenced having some type of N-terminal blockage at the residue following valine-13. The next residues after valine-13 are the first asparagine-glycine sequence in the type A amino acid sequence. Since Edman degradation is stopped by isoaspartyl residues it is possible that deamidation could be occurring here. Such a decrease in signal strength could be used in future experiments to quantitate the proportion of deamidation at a given amino acid residue. The positions of the six asparagine-glycine sequences in type A neurotoxin as well as the eight asparagine-serine sequences are shown in Table 3. These sequences have been shown to be the most labile to deamidation under physiological conditions (Liu, 1992).

Table 3. Positions of asparagine-glycine and asparagine-serine amino acid sequences in type A neurotoxin (adapted from Goodnough and Johnson, 1994, in press).

	Light chain	Heavy chain
Asn-Gly	-15,16-	
	-178,179-	
	-402, 403-	
		-539,540-
		-1032,1033-
		-1243, 1244-
Asn-Ser		-570,571-
		-798,799-
		-935,936-
		-954,955-
		-971,972-
		-1026,1027-
		-1093,1094-
		-1151,1152-

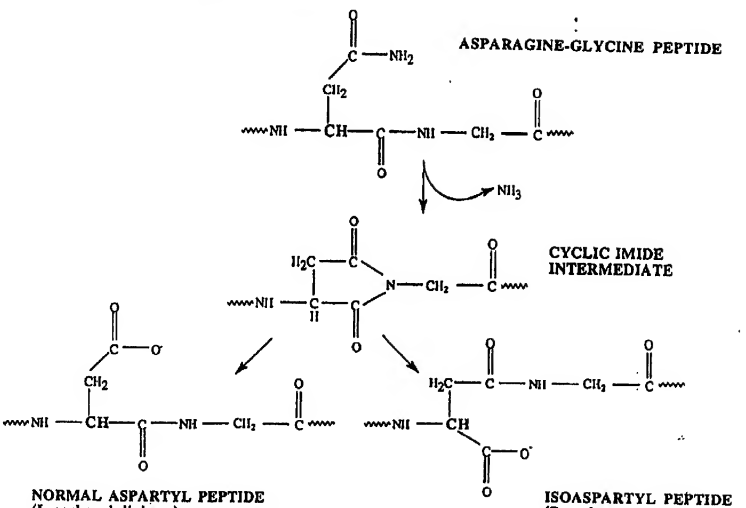
Deamidation requires regional flexibility in the peptide chain for the imide ring structure to form. The formation of the imide ring as well as two cleavage products of the ring structure are shown in Figure 9.

The regional flexibility of the peptide chain of type A botulinum toxin was examined using the method of Ragone et al. (1989) and a five amino acid floating window (D. Aswad, personal communication, 1993). The flexibility plot is shown in Figure 10. The first asparagine-glycine combination at N15G shows a relatively high degree of flexibility indicating that deamidation would be favored. The aspartate-glycine sequence at D141G in the light chain region appears to be very flexible as do the N971S, N1093S, and N1243G sequences of the heavy chain region. Additional research including peptide mapping will be necessary to determine to what degree these sequences are deamidated.

Further support for N-terminal blockage of the light chain sequence was obtained by examination of light chain fragmentation. The sequence determined from the lower molecular weight band of the light chain doublet (50 and 52 kDa) was completely N-terminally blocked after the third cycle of the sequencer. The first two cycles showed residues one and two to be proline and valine, respectively. Residue number three of the 50 kDa band would correspond to the asparagine-14 residue of the intact light chain. This is the first asparagine-glycine sequence in the light chain.

Isoelectric focusing (IEF) is one method of detecting deamidation as the pI shifts accordingly with the loss of amino groups from asparagine residues with the consequent gain of a carboxyl group. It is problematic to detect a change in pI by IEF with purified type A toxin as the molecule is too large to show a charge shift from losses of a minimal number of amino groups (Johnson, et al., 1989b; Liu, 1992). It may be possible in the future to show shifts in IEF of fragments of purified type A and B neurotoxin.

Figure 9. Mechanism of deamidation (adapted from Liu, 1992).



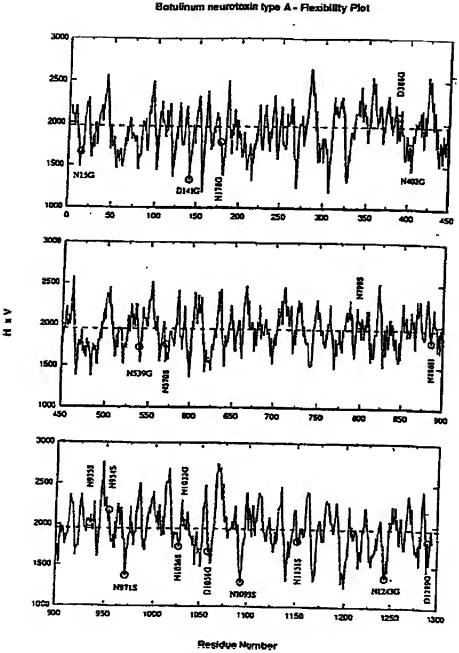


Figure 10. Flexibility plot of type A botulinum neurotoxin using a five amino acid floating window. Regions with higher flexibility have a lower H x V number.

Peptide bond hydrolysis was not observed during initial studies involving type A toxin complex. However, when purified toxin was substituted for the complex, hydrolysis was seen at the aspartate 11-proline 12 linkage as determined by amino acid sequencing. There are at least two reasons why hydrolysis of toxin in the complex was not detected. First, the samples of toxin complex that were electrophoresed after lyophilization and reconstitution were solubilized in dH_2O and the insoluble material separated by centrifugation. When neurotoxin was analyzed, the aggregated material was found to have a higher percentage of hydrolyzed peptides and may have been removed during sample preparation. The second reason hydrolysis was not detected in lyophilized toxin complex is that the migration rates of the fragmented toxin was nearly equivalent to that of two of the non-toxic proteins associated with the toxin in the complex. If the unreduced toxin present in the toxin complex was hydrolyzed at the same point as purified toxin, the lower molecular weight band of the doublet present at 143-145 kDa would run very close to the 118 kDa non-toxic complex protein which may have masked the detection. In the reduced sample, the fragmented light chain running at ca. 50 kDa would overlap the non-toxic complex protein running at about the same molecular weight.

Amino acid sequencing showed that the aspartate-proline bond at the N-terminus of the light chain was cleaved during lyophilization. It has been shown that sodium phosphate buffered systems can change pH during the freeze-concentration step of lyophilization. The pH of a sodium phosphate buffered solution at pH 7 can decrease to 5 during the freezing portion of a lyophilization cycle (Pikal, 1990; Van den Berg, 1966). Additionally, it has been shown that type A neurotoxin is susceptible to acid hydrolysis of the aspartate-proline bonds of the heavy chain at pH values as high as 5 (DasGupta and Tepp, 1991). The short 12 amino acid peptide resulting from cleavage of the N-terminal aspartate-proline bond has a molecular weight of about 1400 daltons. Such a molecular weight correlates well with the observed differences between the 50 and 52 kDa bands. The origin of the

fragment must be the N-terminus of the light chain since the partially hydrolysed, unreduced samples run with molecular weights of 143 and 145 kDa. If the fragment were being hydrolyzed from the C-terminus of the light chain, then the fragments would be running at 50 and 93 kDa without being reduced since the cysteine residue involved in the disulfide bridge connecting the two chains would have been excised. Also, if the fragment originated from the C-terminus of the heavy chain, the light-chain doublet would not be seen in reduced samples, instead the heavy chain would show a doublet upon reduction.

In conclusion, the mechanisms of inactivation of botulinal toxin are complex and varied. These mechanisms involve aggregation, deamidation, and peptide bond hydrolysis. Further study of the inactivation mechanisms will include the following: peptide mapping, isoelectric focusing of purified fragments of the toxin before and after lyophilization and comparison to measurements of isoaspartyl residues in the lyophilized and unlyophilized fragments. Such experiments will give a clearer picture of the events occurring during the freeze-drying process.

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CHAPTER VII

Immune response of rabbits to low doses of type A botulinum toxin.

Abstract

Sublethal doses of various preparations of Clostridium botulinum type A toxin including Botox® and Dysport® were tested in a rabbit model for immunogenicity. Known quantities of various type A toxin preparations were injected over a period of time and the animals serum assayed for antibodies capable of neutralizing a small challenge of purified type A toxin in mice. Animals injected with a total of 18.3 and 18.1 ng of Botox® developed neutralizing antibodies to the toxin in 60 and 63 days. Animals injected with 5.89 ng of crystalline type A toxin complex from our laboratory produced antibodies that neutralized the challenge in 118 days. Animals injected with 0.92 ng of purified type A neurotoxin over 118 days did not produce neutralizing antibodies. Likewise, 5.89 ng of Dysport® administered over 165 days did not produce neutralizing antibodies.

Introduction

Clostridium botulinum is a diverse group of organisms capable of producing a potent proteinaceous neurotoxin. Different serotypes of C. botulinum produce neurotoxins that differ in primary amino acid sequence and thus are antigenically distinct. There are seven antigenically different serotypes currently recognized, A, B, C_1, D, E, F, and G. The organisms producing these toxins catagorized by biochemical and growth requirements (Hatheway, 1990). The toxin causes a flaccid paralysis by binding to the pre-synaptic junction of motor neurons and inhibiting the release of the neurotransmitter acetylcholine (Schantz and Johnson, 1992; Simpson, 1981). The particular muscle or muscle group innervated by this particular motor neuron does not receive the signal to contract resulting in flaccid paralysis. Classically, this condition which is termed botulism has been linked to the consuption of food products which contained the preformed toxin. C. botulinum can also colonize wounds and the infant bowel causing a toxicoinfection (Sugiyama, 1980).

Since the early 1980's, investigations by Drs. Edward Schantz and Alan Scott have led to use of type A toxin for treatment of various spastic muscle disorders as an alternative to surgical techniques. The toxin is used in nanogram quantities and is injected directly into the affected muscle groups. Small enough quantities are used to ensure that the dose is effective but does not spread to other regions of the body. Originally, only a few indications were approved for treatment by the United States Food and Drug Administration including blepharospasm, hemifacial spasm, and strabismus. The use of the toxin to treat other focal dystonias such as spasmotic torticollis, writer's cramp, vocal dystonias, and club foot in children has led to ever increasing use for a variety of neurological disorders. Unfortunately, one of the major side-effects of the use of certain protein pharmaceuticals in high enough quantities is the production of antibodies by the patient to the drug. The presence of circulating antibodies has been demonstrated in

patients receiving large doses of the commecially available toxin (Jankovic and Schwatz, 1991).

We have developed a rabbit model in which repetitive injections of various type A toxin preparations have been given to simulate the treatment of a focal dystonia in order to assess the immunogenicity of the toxin. The model consists of injecting albino rabbits with sub-lethal doses of the toxin over a period of time and assaying the serum of the animals for the ability to neutralize a small but carefully quantitated amount of purified type A toxin. Our results show that the product presently available in the United States is the most antigenic of all the preparations tested to date while purified type A toxin and the product commercially available in Europe are the least antigenic. These results indicate that high specific activity preparations reduce the probability of patients developing neutralizing antibodies.

Materials and Methods

Animals.

Female, ICR mice, 18-22 g (Harlan Sprague Dawley, Madison, WI) were used in toxin and serum titrations. New Zealand, albino rabbits, 4-6 lbs. were obtained from Hazelton Laboratories, Kalamazoo, MI.

Bacterial strains and growth conditions.

The Hall A strain of type A C. botulinum was used to produce crystalline type A complex. This strain was originally obtained from Dr. J. H. Mueller at Harvard University and was further screened for high toxin titers at Fort Detrick, MD by Dr. E. J. Schantz and coworkers. This strain is routinely used for production of type A botulinum toxin due to high toxin titers and the rapid onset of cell lysis (usually within 48 h).

Stock cultures of C. botulinum Hall A were grown statically in 15 ml Hungate tubes containing 10 ml of cooked meat medium + 0.3 % dextrose (CMM, [Difco Laboratories, Detroit, MI]) under an anaerobic atmosphere (80% N_2, 10%CO_2, 10%H_2) at 37°C for 24 h and frozen at -20°C until use. CMM cultures of the Hall A strain gave toxin titers in excess of 10^6 LD_{50}/ml within 48-72 h.

For toxin production, cultures of Hall A were grown statically in 15 liter volumes of toxin production medium (TPM) consisting of 2.0% NZ TT (lot # 9NC29) casein hydrolysate (Sheffield Laboratories, Norwich, NY), 1.0% yeast extract (Difco), and 0.5% dextrose, pH 7.3-7.4, for 5-7 days at 37°C. Cultures of Hall A showed heavy growth in this medium during the first 24-48 h followed by autolysis of the culture which was evident as a clearing and settling over the next 48-120 h.

Type A toxin complex.

Type A toxin complex was purified according to a modification the method of Duff et al. (1957). Briefly, the method involves a series of precipitations and extractions using low pH, ethanol, and crystallizations of the toxin complex for purification from crude culture (Schantz, 1964).

Commercially available type A toxin complex preparations (Botox® and Dysport®) as well as a new type A toxin product currently undergoing clinical trials (ASB) were obtained from Dr. Gary Borodic, Massachusetts Eye and Ear Infirmary, Boston, MA.

Type A neurotoxin purification.

Type A neurotoxin was purified from the associated non-toxic proteins of the complex by a modification of the method of Tse et al. (1982). Toxin complex in 50 mM sodium citrate buffer was eluted from a 1 liter DEAE-Sephadex A50 column (5 cm x 65 cm) and precipitated by addition of 39 g of solid ammonium sulfate/100ml. The precipitated toxin complex was collected by centrifugation (12,000 x g, 5-10°C, 20 min), dialyzed against 25 mM sodium phosphate, pH 7.9, and applied to a DEAE-Sephadex A50 column equilibrated with the same buffer. The binding capacity of this particular matrix under these conditions is 0.9 mg of complex/ml of swollen gel. Various size columns were utilized by applying ca. 90% of the column binding capacity. Toxin was separated from the non-toxic proteins of the complex and eluted from the column with a linear 0-0.5M sodium chloride gradient. Toxin eluted from the column in the first protein peak and fractions which had 260/278 nm absorbance ratios <0.6 were pooled and precipitated by adding 39 g of solid ammonium sulfate/100 mL. Material recovered from the DEAE-Sephadex A50 column at pH 7.9 was further purified by chromatography on SP-Sephadex C50. Precipitated toxin from DEAE-Sephadex A50 columns at pH 7.9 was collected by centrifugation (12,000 x g, 5-10°C, 20 min) and dialyzed against 25 mM sodium

phosphate, pH 7.0. The dialyzed toxin was applied to 25 ml SP-Sephadex C50 in 25 mM sodium phosphate, pH 7.0. Contaminating material did not bind to the column under these conditions. The toxin was eluted with a linear 0-0.25 M sodium chloride gradient.

SDS-gel electrophoresis.

Electrophoresis was performed using a Pharmacia Phast System (Pharmacia LKB Biotechnology, Piscataway, NY) and 12.5% linear pre-cast gels according to the manufacturers instructions. Sample buffer consisted of 75 mM Tris-HCl (Sigma Chemical Co., St. Louis, MO), 5 M urea (Sigma), 5% SDS (Sigma), and 20% glycerol (Sigma), pH 6.8. All samples were boiled for 5-10 min. Some samples were reduced by the addition of 0.5% (w/v) dithiothreitol. Bands were visualized by staining in 0.1% Coomassie brilliant blue R250 in 5:5:2 dH_2O:methanol:acetic acid, destaining by diffusion in 9:3.3:1 dH_2O:methanol:acetic acid. Some gels were silver stained according to the procedure of Hames and Rickwood (1990).

Titration of toxin samples.

Toxin sample titers were estimated using the intravenous method of Boroff and Fleck (1966) in groups of 5 mice per dilution. Titers were determined more accurately using the standardized intraperitoneal method of Schantz and Kautter (1978) with 5 mice per dilution.

Toxin standard.

A toxin standard was prepared using purified type A neurotoxin in 50mM sodium acetate, 2 mg/ml gelatin (Difco), 3 mg/ml bovine serum albumin (Sigma), pH 4.2, according to the method Schantz and Kautter (1978). The standard was stored at 4°C. The toxin standard was titrated according to the method of Schantz and Kautter (1978) using

seven mice per dilution. The standard contained 56 LD_{50}/ml when freshly prepared. When titrated 9 months later in the same fashion, the standard contained 60 LD_{50}/ml which was within the ±15% margin of error given by Schantz and Kautter (1978) for intraperitoneal bioassay of botulinal toxin.

Sub-lethal injection of rabbits.

Rabbits were injected on the days indicated in Table 1. Initially, 0.1 ml injections were made intramuscularly in the hind legs with subsequent 0.1 ml boosts given subcutaneously over the front shoulders. A total of 0.2 ml was given to each animal on each day indicated which amounted to 12-15 LD_{50}/boost. Blood samples were drawn from the central vein in the ear. Serum samples were taken on the same days just prior to injection. Five ml samples of whole blood were incubated on ice for 1 h at which time the serum was separated by centrifugation at 5,000 rpm in a Sorval SS-34 rotor at 4-10°C for 20 min. Samples were kept frozen at -20°C until assayed for type A toxin antibodies.

Enzyme-linked immunosorbent assay (ELISA) of toxin samples.

A modified ELISA was used for determination of the number of nanograms of toxin/ml of the reconstituted commercial products. Sandwich complexes consisting of the toxin preparation being tested, chicken immunoglobulin Y specific for type A toxin, and horse immunoglobulin G specific for type A toxin conjugated to Russell's viper venom factor XA (RVV-XA) activating enzyme were formed in solution. The complexes, consisting of the two different antibodies bound to the toxin, were captured on a microtiter plate coated with rabbit immunoglobulin G specific for chicken immunoglobulin Y. After capture, a mixture of coagulation factors II, V, and X were added. A positive result indicating the presence of toxin generated thrombin due to the presence of RVV-XA. Alkaline phosphatase labelled fibrinogen was added along with polystyrene pegs coated

with fibrinogen. Thrombin caused hydrolysis of fibrinogen to fibrin which resulted in deposition of labelled fibrin onto the polystyrene pegs. The pegs were then removed, washed, and placed in phenolphthalein monophosphate as a substrate for alkaline phosphatase (Doelgast, et al., 1993). The assays were performed by Dr. Mike Roman, Kraft General Foods, Glenview, IL.

Results

Purification of type A toxin complex.

The batch of toxin complex used in these experiments was recrystallized three times and had a specific toxicity of 18 mouse intraperitoneal 50% lethal doses/ng (LD_{50}). The absorbance ratio at 260/278 nm which is used as one measure of purity (Schantz and Johnson, 1992) was 0.52 indicating that the preparation was relatively free of contaminating nucleic acids. SDS-gel electrophoresis demonstrated bands (from top of gel down in kDa) of 145, 118, 48, 35, 29, 22, 21, and 15 which are indicative of type A complex (Figure 1, lane 4) (Johnson and Goodnough, 1993, in press). When the sample was reduced with dithiothreitol the two bands indicative of type A toxin heavy chain (93 kDa) and light chain (52 kDa) were observed (Figure 1, lane 5). For injection into rabbits the type A complex was diluted to 60 LD_{50}/ml in 30mM sodium phosphate, 0.2% gelatin, pH 6.4 (gel-phosphate).

Purification of type A neurotoxin.

Type A neurotoxin was purified to homogeneity (Figure 1, lanes 2 (unreduced) and 3 (reduced)). The 260/278nm absorbance ratio of the preparation was 0.50. The specific toxicity was approximately 96 LD_{50}/ng. For injection into rabbits the type A neurotoxin was diluted to 60 LD_{50}/ml in gel-phosphate, pH 6.4.

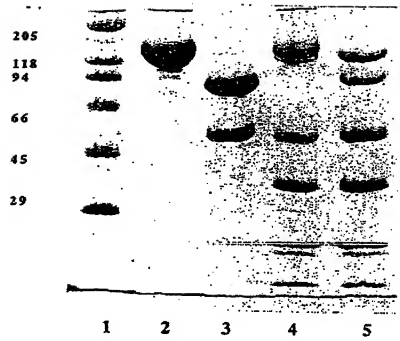

Figure 1. SDS-PAGE analysis of type A toxin preparations. Lane 1, molecular weight markers (in kDa); rabbbit myosin, 205, E. coli ß-galactosidase, 118, E. coli phosphorylase, 94, bovine serum albumin, 66, ovalbumin, 45, carbonic anhydrase, 29, (Sigma Chemical Co., St. Louis, MO), 5-6 µg protein total; lane 2, purified type A neurotoxin, 4-5 µg protein; lane 3, purified type A neurotoxin (reduced with 0.5% (w/v) dithiothreitol), 4-5 µg protein; lane 4, type A toxin complex, 4-5 µg protein; lane 5, type A toxin complex (reduced), 4-5 µg protein.

Titration of Botox®, Dysport®, and ASB.

Botox®, the commercial type A toxin product of Allergan, Dysport®, the commercial product of Porton Down, and ASB were titrated using the method of Schantz and Kautter (1978) with five mice per dilution. Two vials of Botox® had 70 LD_{50}/ml and 66 LD_{50}/ml when reconstituted in 2.0 ml of dH_2O. One vial of Dysport® had a titer of 217 LD_{50}/ml when dissolved in a total of 2.0 ml of dH_2O. This material was diluted to 60 LD_{50}/ml in gel-phosphate, pH 6.4, prior to injection in rabbits. Two vials of ASB contained 69 and 72 LD_{50}/ml when treated in a similar fashion.

Serum titrations.

Rabbit serum was assayed for neutralizing antibodies by combining 0.1 ml of the toxin standard along with 0.5 ml of a serum dilution and 0.6 ml of gel-phosphate, pH 6.4. The solution was incubated at room temperature for 30-60 min with occasional mixing by inversion. One-half ml of the solution was injected intraperitoneally into each of two mice. Mice were observed for signs of botulism for a period of four days. Serum samples which neutralized type A toxin were diluted in two-fold increments and the neutralization assay repeated in the same fashion to determine the neutralizing titer of the serum.

Serum titrations.

Pre-immune serum samples were all negative for antibodies capable of neutralizing type A toxin (Table 1). Antibodies were not detected up to day 118 for the animal given type A toxin complex and day 60 and 63 for the Botox® animals. Titers of type A antibodies continued to rise in the second Botox® animal (Botox® II) with repeated boosts of the toxin. Numbers in brackets following a day indicated the highest two-fold dilution which neutralized 5.6 LD_{50} of the type A toxin standard.

Table 1. Immune response of rabbits to sub-lethal doses of type A botulinal toxin.

Toxin sample	Days of injection/titration[a]	Total ng given	Specific toxicity[b]
A neurotoxin	0, 29, 42, 56, 69, 88, 107, 118 (no antibodies detected)	0.92	96
A complex	0, 29, 42, 56, 69, 88, 109, 118 (1:2)	5.12	18
Botox®			
I*	0, 21, 35, 49, 60 (1:1)	18.3	4.3
II*	0, 21, 35, 49, 63 (1:1), 77 (1:2), 84 (1:4)	25.4	4.3
Dysport®	0, 21, 32, 46, 60, 67, 81, 95, 109, 123, 137, 151, 165 (no antibodies detected)	5.89	25.6
ASB	0, 21, 35, 49, 60 (no antibodies detected)	4.08	17.3

*Two separate animal trials designated I and II are represented.

[a]All antibody samples were titrated against 5.6 LD_{50} of purified type A neurotoxin according to the following: 0.5ml serum + 0.1ml containing 5.6 LD_{50} type A toxin + 0.6ml gel-phosphate, pH 6.4. The solution was incubated at room temperature for 30-60 minutes. Two mice per two fold dilution were injected intraperitoneally with 0.5ml of serum + toxin mixture. Dilutions which neutralized the toxin challenge are indicated in brackets. [b]LD_{50}/ng.

ELISA results.

The ELISAs performed on Botox®, Dysport®, and ASB which when corrected for the known concentration of ASB (8.15 ng/vial) gave values of 36.3 ng/vial of Botox® and 16.95 ng/vial of Dysport®. It should be noted that both ASB and Botox® are labelled to contain 100 LD_{50}/vial while Dysport® is labelled to contain 500 LD_{50}/vial. These results indicated that Botox® had an average specific toxicity of 4.3 LD_{50}/ng, Dysport® had 25.6 LD_{50}/ng, and ASB had an average specific toxicity of 17.3 LD_{50}/ng after reconstitution.

Discussion

The likelihood of antibody formation in patients treated with Botox® seems to depend on the amount of material used to treat the particular disorder as well as the number of treatments received by each patient (Jankovic and Schwartz, 1991; Borodic et al., 1991). Patients who receive higher doses of toxin on a more frequent basis are those who are more likely to produce neutralizing antibodies to the toxin. In particular, those patients with spasmodic torticollis are at high risk due to the large doses of toxin which are spread over a large area. We have used a rabbit model for testing the immunogenicity of various toxin preparations in a manner similar to their intended use, i.e. by injection of sub-lethal doses on a repetitive basis. In this study, neutralization of a small but accurately quantitated amount of toxin gave greater sensitivity in detecting antibodies than previously reported (Hatheway et al., 1984). In our assay, as little as 0.0012 IU/ml of type A specific antibodies could be measured.

We have compared the two currently available commercial products (Botox® and Dysport®) to two type A toxin preparations made at the Food Research Institute and one type A toxin preparation currently undergoing clinical trials (ASB). The two preparations made at the Food Research Institute had specific activities that were 18 LD_{50}/ng for type A toxin complex and 96 LD_{50}/ng for purified type A neurotoxin. Botox® has been reported to contain 2.5 LD_{50}/ng which gives it the lowest specific activity among those tested. Our results of 4.3 LD_{50}/ng were slightly higher than this value. The data from the amplified ELISA assay correlates well with this estimate as 2.5 LD_{50}/ng x 36.3 ng = 90.75 LD_{50}/vial. However, the average of the toxin titrations of the individual vials gives a specific toxicity of 3.7 LD_{50}/ng. Using the same approach, the specific toxicity of Dysport® used in this study was estimated to have a specific activity of 434 LD_{50}/vial / 16.95 ng/vial which equals 25.6 LD_{50}/ng. This is close to the specific activity expected

with chromatographically purified type A toxin complex that has been lyophilized under conditions allowing recovery of 75-85% of the active toxin (Goodnough and Johnson, manuscript in preparation).

Lesser quantities by weight of the preparations with higher specific activity were injected (see Table 1). On a weight basis, 10 LD_{50} of purified type A neurotoxin with a specific toxicity of 96 LD_{50}/ng was equal to 104 pg. Ten LD_{50} of the type A complex with a specific toxicity of 18 LD_{50}/ng which was purified at the Food Research Institute was approximatly 550 pg. Since less toxin is being given, the immune system of the animal is exposed to less of the antigenic substance. Additionally, it has been shown that the non-toxic components of the complex including those with hemagglutinating properties are more antigenic than the toxin itself (Sakaguchi et al., 1974; George Doellgast, personal communication). Hence, the non-toxic components could be acting as adjuvants stimulating a stronger immune response than a more purified toxin. An additional adjuvant present in all vials of lyophilized toxin is toxoid of the specific toxin formed during handling and lyophilization of the toxin itself. This problem can be reduced by up to 10 fold by utilizing the appropriate formulation and drying conditions (Goodnough and Johnson, 1992).

Possible solutions to the problem of antibody formation in patients being treated for spastic muscle disorders include: a). use of botulinal toxins with higher specific activities, including those which have been chromatographically purified from the more antigenic non-toxic and hemagglutinating fractions, and b). implementation of drying processes which result in a higher percent recovery of active toxin to minimize the formation and presence of toxoids of the toxin. Narrowing the range of permitted LD_{50}'s per vial by the Food and Drug Administration from $\pm$ 30% (which allows for up to 130 LD_{50} of toxin in a given vial) to some lower value (e.g. 10-20%) would reduce the chance of inadvertant administration of excess toxin. Such an excess could not only further stimulate the

production of antibodies by a patient but could also result in dangerous side-effects such as ptosis and inadvertant paralysis of muscles at sites distant from the injection.

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CHAPTER VIII

Conclusions and Future Prospects

Conclusions and commentary.

There is a need for standardization regarding botulinal toxins as used in the medical field. This became very evident during the Interagency Botulinal Research Council Committee conference on botulinum toxins held in Madison, WI in December of 1993. The current definition of a unit of botulinum toxin used pharmaceutically is one unit of toxic activity is equivalent to 1 mouse intraperitoneal 50% lethal dose or 1 LD_{50}. There are a couple of difficulties, however, in using the term 'unit' when speaking of botulinum toxin. It has become apparent that some researchers and clinicians who use the toxin on humans do not have a full understanding of this convention. Frequently, the unit of toxin is written about and spoken of as an international unit. An international unit is a description of a quantity of anti-toxin not toxin. One international unit of antitoxin is equivalent to 10,000 LD_{50} of types A, B, C, D, and F toxin. Also, one international unit of antitoxin is equivalent to 1,000 LD_{50} of type E toxin. A standard unit needs to be defined for biological toxicity of botulinal toxin including the ng of toxin and non-neurotoxin proteins in each preparation.

An additional point of confusion is the definition of a 50% lethal dose of botulinum toxin. The dose is usually referenced in terms of the amount needed to kill half of a population of white mice. However, the goal in treating patients with botulinal toxin is not to kill half of the population but rather to treat a given muscle group with enough toxin to elicit the desired response. Another biological unit has been proposed, the median paralysis unit, which is less than an LD_{50} but more difficult to quantitate as it currently involves injecting an amount of toxin into hind limb muscles of mice and rats and observing the regional paralysis and duration of the paralysis. More work will need to be done on this idea if it is going to be generally accepted and used consistently by independent laboratories. It will be necessary to develop a simplified procedure with a definite end point if it is going to supersede the LD_{50} as a unit of measurement. An

additional complication is the fact that the various toxin serotypes have different durations of action. There is even evidence that different strains within a given serotype have differing durations of action. So, for a given amount of denervation needed to achieve relief from a particular dystonia, the amount of toxin used will be different for each serotype used and possibly for each strain in an individual serotype. Only serotype A produced by one strain is currently being used in the United States. It will be necessary to develop other serotypes and strains since an increasing number of patients are developing neutralizing antibodies to the current product.

There is a need for an alternative botulinal toxin product for treating spastic muscle disorders in the United States. The current product has a very low specific activity for type A toxin complex which when coupled with the present formulation used in the drying process (inclusion of sodium chloride and alkaline pH) results in a product with less active toxin than inactive toxoid. The consequences of this are that the patients being treated with the material are developing antibodies at an alarming rate. A different product that was designed to alleviate the above concerns would certainly be an improvement. The use of purified neurotoxins has been shown here to be possible in a pharmaceutical product. The use of such material in concert with a drying excipient that would allow the shipment and storage of the material at room temperature would reduce the major drawbacks with extended use of botulinal toxin as a therapeutic.

Future considerations that may spring from some of this research include some of the following: 1. new methods of determining the antigenicity of individual protein pharmaceutical preparations using *in vivo* methodology, 2. further investigation into the mechanisms of inactivation of protein pharmaceuticals during preparation, handling, and storage, and 3. development of additional botulinum toxin serotypes and combinations of serotypes to treat patients with focal dystonias.

The notion that the toxin and non-toxic binding proteins themselves are capable of passing through the intestinal barrier may be a novel method of delivery of other pharmaceutical compounds. This possibility was not explored in this thesis but may warrant further attention. Also, limited work has been done regarding the development of chimeric toxin molecules that exploit the specificity and binding affinity of the heavy chain of botulinum toxin. It may be possible to develop other pharmaceuticals that are targeted to the terminal neurons using this specificity.

Drug delivery is one aspect of botulinal toxin therapy that has not been seriously addressed. Possible future work could include the development of a sustained release formulation that would be similar to the contraceptive Norplant where the pharmaceutical is released slowly through diffusion from an inert carrier such as latex. Hypothetically named "Toxplant", the advantages of using such a delivery would be that the treatment intervals could be extended to many months or even years. The current treatments involve intramuscular injections every 2-3 months which can be painful. Further, very low amounts of the toxin would diffuse out from such a delivery vehicle maintaining the desired effect on the neuro-muscular junction while avoiding stimulation of the immune system by only releasing very small quantities at any given time.

Since there are different target proteins for each toxin serotype, combining two or more toxin serotypes may allow the use of much lower quantities of toxin through synergistic effects. In the same vein, the use of a combination of botulinal neurotoxin and a chimeric toxin that uses the binding specificity and affinity of botulinal neurotoxin heavy chain and the active portion of a toxin that would slow or inhibit the resprouting of nerve terminals (the primary reason for retreating patients) would be advantageous. Such a chimeric toxin might be one that inhibits protein sysnthesis such as some of the ADP-ribosylating toxins including diphtheria toxin, ricin, and botulinal toxins C_2 and C_3.